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A COMPARISON OF SHOCK DUE TO ENDOTOXIN WITH ANAPHYLACTIC SHOCK

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THE cellular component of gram-negative bacteria, designated as endotoxin, is a lipoprotein-carbohydrate complex situated on or near the surface of the bacterial cell.[1, 2] This constituent is one of the features that distinguishes gram-negative bacteria from gram-positive organisms. There is no evidence that invasiveness or virulence of the bacterial cell is closely related to the surface endotoxin, but the dissemination in the body of endotoxin from gram-negative bacteria having attenuated virulence can provoke a serious, and even fatal, illness. Not infrequently, bacteremia due to gram-negative organisms has caused irreversible shock.[1-7] The increasing clinical significance of shock due to endotoxin has led to extensive inquiry into the underlying mechanisms involved.[8-10] In cooperation with the Departments of Physiology and Surgery, the mechanism of this type of shock has been studied intensively and the results have been reported.[11-18]

During the progress of the investigations on the mechanism of shock induced in the dog by endotoxin, it became increasingly apparent that the type of shock produced by a single intravenous injection of endotoxin—designated as anaphylactoid shock—was remarkably similar to the reported sequence of findings, known as anaphylactic shock, in sensitized dogs given a second injection of specific antigen. These similarities were of considerable interest because of studies in this clinic on the role of brucella endotoxin in the pathogenesis of illness. Experimental and clinical observations have indicated quite clearly

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that illness arises not only because of the nonspecific, intrinsic toxicity of the brucella endotoxin, but also because of the appearance of specific hypersensitivity of host tissue to the endotoxin.[19-22] In some animal species, at least, the undesirable effects of an initial dose of endotoxin could be prevented by the administration of an adrenocorticotropic hormone or by adrenal corticosteroids, and the toxic manifestations of hypersensitivity to brucella endotoxin in the human being could also be subdued by the steroids.[23-24] Patients having acute brucellosis exhibit a remarkable sense of well-being shortly following steroid therapy.

Certain similarities between the physiologic changes produced by endotoxin and by serum anaphylaxis were recognized by Anderson and Rosenau[25] as early as 1909, only 7 years after the original description of anaphylaxis by Portier and Richet.[26] They predicted at that time that further studies of anaphylaxis would offer an explanation for the poisonous nature of endotoxin. However, the fundamental features of this relationship have received only sporadic attention. Bronfenbrenner[27] probed this relationship for many years and offered the provocative explanation that a fundamentally common chain of events is set off in anaphylactoid and anaphylactic activity. Nevertheless, the nature of the initial injury which triggers this chain of events is not understood. The present status of the pertinent theories has been reviewed by Burdon.[28] Since more information is needed to clarify any basic relationship that may exist between these reactions, we desire to compare our results of investigations on the response of the dog to endotoxin with the documented information on anaphylaxis in the dog.

METHODS

Preparation and Activity of Endotoxin.—Endotoxins from different species of gram-negative microorganisms have similar physiologic effects. In the present experiments, endotoxins from *Brucella melitensis, Escherichia coli, Salmonella typhosa,* and *serratia marcescens* were used.* When differences in physiologic potency of the various lots were adjusted, each of these endotoxins produced the characteristic effects in dogs which were indistinguishable from one another.

In the initial experiments to be described, *E. coli* or *Br. melitensis* endotoxin of the Boivin type was employed.[1] On the basis of exploratory experiments, a single injection of 5 mg. of *E. coli* endotoxin or 10 mg. of *Br. melitensis* endotoxin per kilogram body weight was used since these amounts regularly produced shock when injected intravenously in the dog.

Because of the large and costly amounts of endotoxin that were needed to produce shock in the dog, the response to less purified preparations was subsequently explored. A preparation of dried bacterial bodies was finally selected, which was made from *E. coli* cells grown on synthetic protein-free media according to a modification of a technique described by Braude and associates.[29] The organisms were harvested in a Sharples centrifuge and washed with alcohol and acetone. The bacterial bodies were dried and then suspended in saline to give a concentration of 50 mg. per ml. A response which was indistinguishable from that produced by the highly purified endotoxins was regularly produced in the dog following the injection of 0.3 ml. per kilogram of this preparation.

The possibility that the anaphylaxis-like reactions to endotoxin were the result of protein contaminants in the endotoxins, derived from the bacterial media, was excluded by the use of synthetic, protein-free media in the preparation of both purified and "crude" *E. coli* endotoxin. Moreover, the highly purified fractions from *S. marcescens* and *S. typhosa* were essentially protein-free.

*Endotoxin from *S. typhosa* was supplied by Dr. Dennis Watson of the University of Minnesota and from *S. marcescens* by Dr. M. J. Shear, National Cancer Institute.

RESULTS

Clinical Manifestations.—The initial observations were made with purified endotoxins from *E. coli* or *Br. melitensis,* which were injected intravenously into healthy adult dogs.[11, 12, 16] Puppies were avoided because shock was not uniformly produced in the immature animal. Shortly after the injection was completed, the dog had a forceful, well-formed bowel movement. Two to three minutes after injection, pruritus, hyperpnea, agitation, vomiting, and diarrhea were manifested. The animal began to shiver markedly and the blood pressure fell progressively. The dog became apathetic, resting quietly in a corner but frequently he would walk in ataxic fashion for short distances, dragging his hind legs as if paralyzed. Over the first hour, gagging and tenesmus were prominent and this was subsequently followed by profuse bloody vomitus and bloody diarrhea. A gradual rise in rectal temperature from approximately 39° to 42° C. usually occurred during the first 3 hours. Between 3 to 5 hours, the animal usually lapsed into coma, and death occurred between 12 to 24 hours after the injection of endotoxin.

In their earliest writings, Portier and Richet[26] noted practically identical clinical events in sensitized dogs after the second or precipitating injection of antigen. The prominence of gastrointestinal manifestations with bloody vomitus and diarrhea and the profound changes in circulation leading to shock were subsequently confirmed by many other students of anaphylaxis. A minor point of difference between anaphylaxis and the reaction due to endotoxin is the elevation in body temperature which usually follows endotoxin but not serum anaphylaxis.[30, 31]

Pathologic Morphology.—Following death of the animal from endotoxin shock the most prominent morphologic changes are found in the gastrointestinal system. Severe congestion of the liver, submucosal bleeding, and epithelial slough of the surface of large portions of the small intestine, and marked edema of the gall bladder were quite uniformly present. Necrosis of liver cells was noted in some animals which survived for longer than 12 hours.

Essentially the same morphologic alterations have been observed in the gastrointestinal tract of dogs following anaphylactic shock.[31-33] The findings in both states are probably to a large extent the result of severe engorgement and vascular stasis occurring in the portal venous system.

Hemodynamic Effects.—In further studies in dogs anesthesized with pentobarbital and given endotoxin, arterial and venous pressures were measured.[17, 18] The arterial pressure began to fall precipitously less than one minute after injection of the endotoxin was completed. The portal vein pressure increased up to 4 times within the first 5 minutes but returned to near its former value after 10 to 20 minutes There was partial recovery of the arterial pressure during the first hour but subsequently the blood pressure again fell to lower values from which it did not ordinarily recover (Fig. 1). The immediate fall in arterial pressure and the marked rise in portal vein pressure were traced to the accumulation of large amounts of blood in the liver and intestine. This was presumably caused by transient obstruction to venous outflow from the liver. The removal of large amounts of blood from active circulation because of this

pooling in the portal system resulted in a critical reduction of the quantity of blood returned to the heart. A marked fall in cardiac output occurred and this was subsequently shown to be the cause of the initial fall in blood pressure.

When the liver was excluded from the circulation by hepatectomy, by evisceration with ligation of the portal vein and hepatic artery, or by portal-cava anastomosis with hepatic artery ligation, no immediate fall in blood pressure followed the injection of endotoxin.[16] These experiments clearly demonstrated that the presence of the liver is essential for this initial collapse in blood pressure.

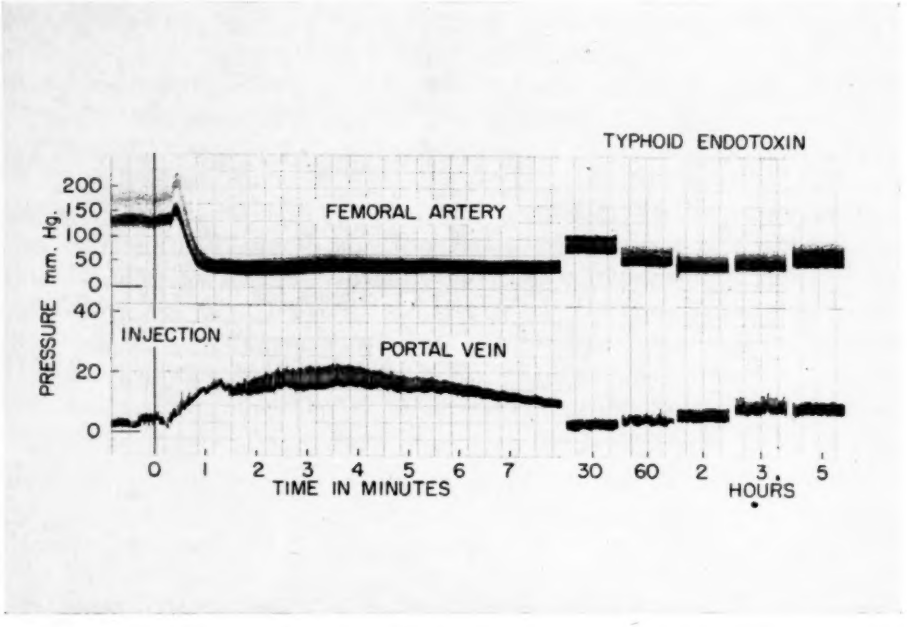

Fig. 1.—Arterial and portal vein pressure curves following administration of *S. typhosa* endotoxin to the dog. The pressures were determined with strain gauge manometer systems with appropriate damping and a polyviso recorder.

In anaphylactic shock a very similar fall in arterial pressure and elevation in portal vein pressure occurs within the first minute following injection of the antigen to which the animal has been sensitized.[34-36] This fall in arterial pressure has likewise been traced to pooling in the portal system and inadequate cardiac filling.[35-37] Manwaring[23] clearly demonstrated that exclusion of the liver from the circulation prevented anaphylaxis, and the parallel situation with endotoxin further emphasizes the fundamental similarity in hemodynamic effects in the two reactions.

Relationship of Shock to Quantitative Changes in Blood Cells, Platelets, and Blood Sugar.—The injection of endotoxin was followed by a rapid and profound fall in the number of white blood cells. A progressive reduction in the percentage of neutrophils with a relative increase in lymphocytes occurred during the first 90 minutes but the maximal fall in total leukocytes had already occurred during the first 15 minutes (Table I). A corresponding and also very

marked decline in the number of platelets took place during the first 5 to 15 minutes (Table II). There was a very close relationship between the abrupt fall in total leukocytes, the decrease in platelets, the drop in arterial pressure, and the transient elevation of the portal vein pressure (Fig. 2). As the leukocyte and platelet counts gradually increased during the 3 hours that followed, a large number of immature red blood cells appeared in the peripheral circulation (Table II).

TABLE I. QUANTITATIVE CHANGES IN TOTAL LEUKOCYTES, NEUTROPHILS, AND LYMPHOCYTES AT VARIOUS PERIODS AFTER INJECTION OF E. COLI OR BR. MELITENSIS ENDOTOXIN INTO DOGS ANESTHETIZED WITH PENTOBARBITAL*

	CONTROL		TIME IN MINUTES AFTER INJECTION OF ENDOTOXIN					
	BEFORE ANESTHESIA	AFTER ANESTHESIA	5	15	30	45	90	180
Total leukocytes cells/mm.^{3}	15,180 (5)	14,188 (4)	1,525 (2)	1,710 (5)	1,925 (4)	2,420 (5)	2,520 (5)	4,900 (3)
Neutrophils per cent	80.0 (5)	80.1 (4)	70.1 (2)	62.4 (5)	66.1 (4)	62.2 (5)	52.2 (5)	73.1 (3)
Lymphocytes per cent	18.1 (5)	17.1 (4)	28.1 (2)	35 (5)	30.3 (4)	35.1 (5)	43.4 (5)	24.2 (3)

*Blood samples were obtained from the femoral vein at the indicated times and the counts were done in duplicate. The mean values and the number of dogs (indicated by parentheses) on which each value is based are shown.

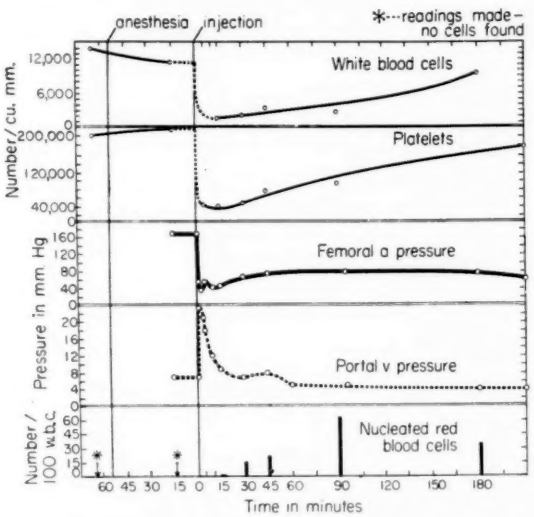

Fig. 2.—Representative hematologic changes and the related arterial and portal vein pressure responses before and following injection of crude *E. coli* endotoxin in the dog.

There was surprisingly little change in the venous hematocrit during the initial 30 minutes. This was in harmony with our earlier observation that pooling of blood in distended veins and not leakage of plasma accounted for the initial hemodynamic disturbance produced by endotoxin.[18] A slight elevation in the concentration of blood glucose during the first 15 minutes was followed by a steep fall during the ensuing 3 hours (Table II and Fig. 3).

The effects of endotoxin on blood cells, platelets, and blood sugar have been well described by other investigators.[38-41] The purpose of including these values

in the present series of experiments was to determine the time relationship between these changes and the sudden fall in blood pressure. It will be noted that leukopenia and thrombocytopenia are closely associated with the sudden appearance of shock.

The close similarity between these hematologic changes and those that follow anaphylactic shock in the dog are noteworthy. Biedl and Kraus[34] first described leukopenia in anaphylactic shock and Webb[42] noted that the abrupt fall in leukocytes occurred within 2 minutes following injection of the shocking

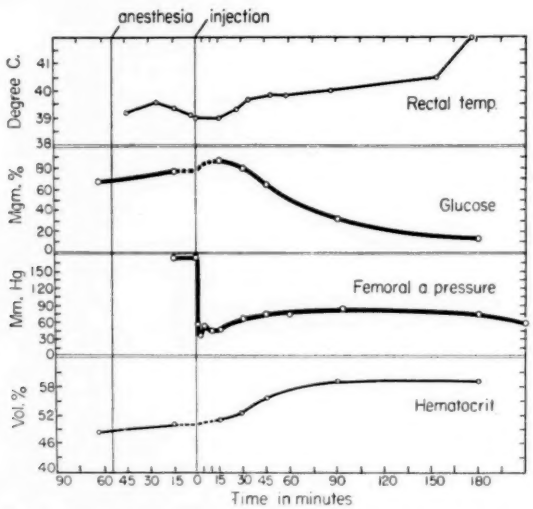

Fig. 3.—Changes in the hematocrit, concentration of blood glucose and rectal temperature, and their relationship to the fall in arterial pressure produced by crude *E. coli* endotoxin.

TABLE II. QUANTITATIVE CHANGES IN THE PLATELET COUNT, NUMBER OF NUCLEATED RED BLOOD CELLS, HEMATOCRIT, AND BLOOD GLUCOSE CONCENTRATION AT VARIOUS PERIODS AFTER INJECTION OF E. COLI OR BR. MELITENSIS ENDOTOXIN INTO DOGS ANESTHETIZED WITH PENTOBARBITAL*

	CONTROL		TIME IN MINUTES AFTER INJECTION OF ENDOTOXIN							
	BEFORE ANESTHESIA	AFTER ANESTHESIA	5	15	30	45	90	120	180	210
Platelet Count No./mm.³	217,000 (5)	242,800 (6)	55,300 (5)	40,500 (6)	62,200 (5)	86,700 (5)	96,100 (4)	75,000 (2)	96,250 (2)	177,000 (1)
Nucleated red blood cells No./100 leukocytes	0 (5)	0 (4)	1 (2)	8 (5)	14 (4)	35.4 (5)	53.5 (5)		30 (3)	
Hematocrit Vol. per cent	51.4 (5)	46.8 (4)	53.1 (2)	50.9 (5)	50 (4)	51.7 (5)	53.6 (5)		55.3 (3)	
Blood Glucose Mg. per cent	69.4 (5)	81.1 (5)	80 (1)	90 (5)	81 (4)	80.2 (5)	76.4 (5)		48.1 (3)	

*Blood samples were obtained from the femoral vein and determinations were done in duplicate. The mean values and the number of dogs (indicated by parentheses) on which each value is based are shown.

dose of antigen. However, recovery of the leukocyte count and the development of leukocytosis apparently proceed somewhat more rapidly after anaphylaxis than after endotoxin.[42, 43] The rapid development of thrombocytopenia after endotoxin is also a constant finding in anaphylactic shock.[44]

Blood Coagulation.—The coagulability of blood was measured before and at intervals after the injection of endotoxin, using a modification of the Lee-White method.[45] No significant change in the coagulation time was detected (Table III). Franke[46] had also noted no consistent change in clotting time in dogs after the injection of large doses of endotoxin derived from *S. marcescens.*

TABLE III. COAGULATION TIMES (MODIFIED LEE-WHITE METHOD) AT VARIOUS PERIODS AFTER INJECTION OF ENDOTOXIN INTO DOGS

NUMBER AND SEX	WT. (KG.)	ENDOTOXIN	COAGULATION TIME IN MINUTES								
			CONTROL		TIME (MIN.) AFTER INJECTION OF ENDOTOXIN						
			BEFORE ANESTHESIA	AFTER ANESTHESIA	5	15	30	45	60	90	180
1 M	25.4	*E. coli* (crude)	9.0	8.5		8.0	9.0		6.0		7.5
2 M	15.0	*E. coli* (crude)	6.5	13.0		10.0	12.5	10.5		8.5	9.0
3 M	23.0	*Br. melitensis* (*purified*)	9.0	8.5		15.5	14.0	19.5		15.5	11.0
4 F	12.0	*E. coli* (*purified*)	7.0		8.5	8.5	6.5	8.0		9.5	
5 M	23.5	*E. coli* (crude)	6.0	6.5		6.0	6.5	9.5		8.0	6.0
Mean			7.4 (5)	9.1 (4)	8.5 (1)	9.6 (5)	9.7 (5)	11.9 (4)	6.0 (1)	10.4 (4)	8.4 (4)

The prolongation of coagulation time due to liberation of heparin is a consistent and well-recognized feature of anaphylactic shock.[34, 47, 48] The lack of evidence that heparin is liberated following endotoxin may constitute a significant difference between anaphylactic shock and the shock produced by endotoxin.

Role of the Central Nervous System.—The characteristic hemodynamic and pathologic changes that follow the injection of endotoxin were not prevented in dogs after cervical chordotomy, with and without vagotomy.[11] A prompt fall in blood pressure and elevation of the portal vein pressure also occurred in decapitated animals. Using a modification of the cross-circulation method of Penner and Klein,[8] which has been presumed to separate the cephalic and body circulation, shock and the classic morphologic changes could not be related to primary activity of endotoxin in the cephalic circulation. These experiments did not support the widely accepted thesis that shock results primarily from the direct effect of endotoxin on the brain.[8]

The earliest workers studying anaphylactic shock also postulated that shock was due to profound chemical effects on the central nervous system. When, in 1909, Anderson and Rosenau[25] compared the manifestations of anaphylaxis and endotoxin, they proposed that both were the result of overwhelming toxic effects on the central nervous system. In refutation of these arguments, Pearce and Eisenbrey[49] demonstrated that decapitation and mechanical destruction of

the spinal cord of the dog did not protect against anaphylactic shock. Anesthesia and sedation may mask the systemic symptoms of anaphylactic shock but the essential characteristics of the reaction are not modified.[34] Our observations in unanesthetized animals and in dogs following pentobarbital anesthesia also demonstrated that vomiting, diarrhea, and minor clinical signs were prevented, but the profound hemodynamic and morphologic alterations were unchanged by anesthesia. The information at hand indicates that the initial reactions produced by anaphylaxis or by endotoxin in the dog are not primarily the result of a direct action on the central nervous system.[11, 34]

*Histamine Liberation.**—Present knowledge of the role played by histamine in anaphylactic shock is incomplete. However, following the studies of Dragstedt and his group,[50-52] liberation of histamine was recognized as a constant feature of anaphylactic shock in the dog. It was, therefore, of considerable interest to determine whether histamine is also released following the injection of endotoxin in dogs.

Method of Assay.—Bio-assay for histamine was performed with a muscle chamber using guinea pig ileum.[53] A freshly removed segment of intestine, approximately 3 cm. in length, was suspended in a 5 ml. chamber filled with Ringer-Tyrode solution. Oxygen was continuously bubbled into the chamber and the system was maintained in a 37° C. water bath. The lower end of the gut was secured by means of a hook to the bottom of the chamber and the upper end was fastened to the free end of a cantilever beam. The initial tension was adjusted to between 0.5 to 1.0 Gm. and subsequent changes in length of the gut were detected by means of a pair of strain gauges cemented to the beam. Recording of the deflection was made on a Sanborn recording system.

With alternate injections of standard quantities of histamine and 0.3 ml. of heparinized plasma, it was possible to determine the quantity of "histamine-like" substance in plasma. The chamber was washed two times with prewarmed Ringer-Tyrode solution between each determination.

TABLE IV. HISTAMINE-LIKE SUBSTANCE DETECTED IN PLASMA OBTAINED FROM THE SUPRADIAPHRAGMATIC INFERIOR VENA CAVA AT INTERVALS AFTER THE INJECTION OF ENDOTOXIN

DOG NO.	WT. (KG.)	SEX	ENDOTOXIN	CONTROL	MICROGRAMS PER ML. PLASMA (MINUTES) 1	2	3	4	5	10	15	20	30	60
1	10.2	M	*Br. melitensis* (purified)	0	0.22	0.13	0.04	0	0	0	0			
2	23.0	M	*E. coli* (cells)	0	0.13	0.04	0	0	0	0	0			
3	9.8	M	*E. coli* (cells)	0	0.03	0.09	0.03	0	0	0	0	0		
4	17.8	F	*E. coli* (cells)	0	0.64	0.39	0.36	0.13	0.08	0	0		0	0

In initial trials, samples of heparinized plasma from the femoral vein of dogs were analyzed before and at intervals after injection of endotoxin. No increase of "histamine-like" substance could be detected in these samples from the dog in a state of shock. When samples were collected through a cardiac catheter, which had been carefully advanced into the superdiaphragmatic inferior vena cava to a site near the inflow of the hepatic veins, "histamine-like" substance was readily detected. The maximum response occurred within one to two minutes after injection of endotoxin and coincided with the period of precipitate fall in the arterial pressure. Between the third and fifth minute, the material had again disappeared from the plasma (Table IV and Fig. 4).

*We are indebted to Mr. R. J. Stish of the Department of Physiology who collaborated in these studies.

A sharp rise and rapid fall of blood histamine was also noted by Code[54] in his studies of anaphylaxis in the dog. The explosive liberation of histamine was likewise shown to be closely related to the onset of hypotension, elevation of the portal vein pressure, and pooling in the viscera. The present findings reveal a significant point of similarity between anaphylactic shock and the reaction to endotoxin.*

Effect of Adrenocortical Hormones.—The therapeutic value of adrenocortical hormones in allergic diseases is well recognized, but reports on the effectiveness of ACTH and cortisone in preventing anaphylactic shock in animals are contradictory.[55-57] However, it would appear that at least partial protection can be demonstrated if the intensity of the anaphylactic reaction is suitably controlled.[57] We have observed that these hormones clearly protect against the lethal effects of endotoxin in the mouse, rat, and rabbit.[20, 58] Ebert and associates[10] could not prevent shock in the dog with meningococcus toxin by pretreating animals with cortisone. In view of the important theoretical and therapeutic implications derived from a knowledge of the effects of corticosteroids on shock produced by endotoxin, this problem was re-explored.

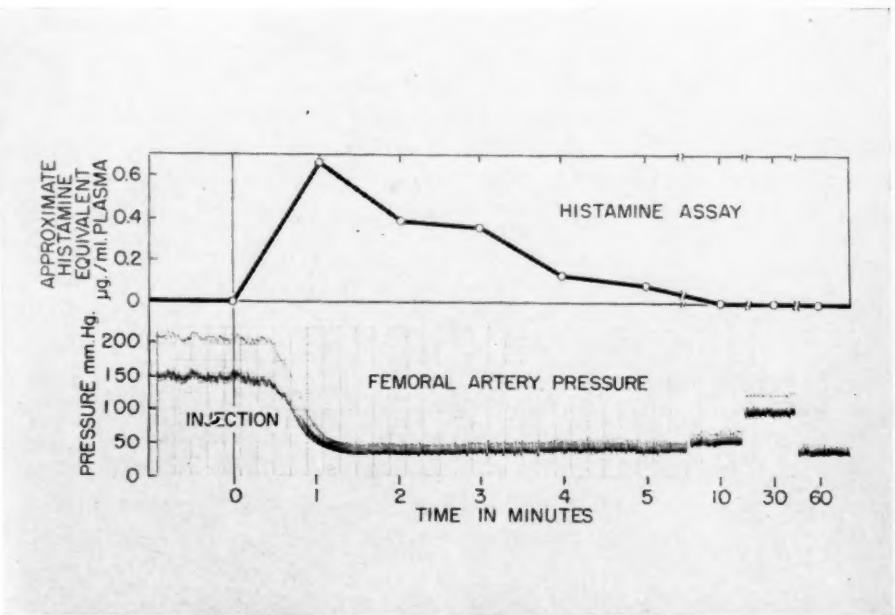

Fig. 4.—Relationship of the amount of histamine-like substance in inferior vena cava blood of the dog to the arterial pressure following injection of crude *E. coli* endotoxin.

Method of Study.—One of a pair of dogs was pretreated with large doses of hydrocortisone hemisuccinate† or prednisolone-21-hemisuccinate† which were injected intravenously 5 to 60 minutes before the endotoxin. Observations on the treated and an untreated control dog were made simultaneously and the experimental animals were so chosen that weight,

*Recent investigations indicate that serotonin, as well as histamine, is liberated into the blood during anaphylaxis in the rabbit (see T. P. Waalkes, H. Weissbach, J. Bozicevich, and S. Undenfriend, J. Clin. Invest. **36**: 1115, 1957).

†Supplied through the courtesy of Dr. C. J. O'Donovan, The Upjohn Company, Kalamazoo, Mich.

TABLE V. EFFECT OF PRETREATMENT WITH ADRENAL STEROIDS ON THE SURVIVAL TIME OF 12 PAIRS OF DOGS AFTER INJECTION OF ENDOTOXIN

	EXPERIMENTAL ANIMAL						CONTROL ANIMAL			
NO.	WT. (KG.)	SEX	THERAPY	TIME PRECEDING ENDOTOXIN (MINUTES)	OTHER THERAPY	SURVIVAL TIME (HOURS)	WT. (KG.)	SEX	SURVIVAL TIME (HOURS)	COMMENT
1	14.5	F	100 mg. Fh 100 mg. Fh (I.M.)	7	None	15½	10.0	F	2½	Anesthetized
2	12.5	F	100 mg. Fh 25 mg. Fh (I.M.)	8	50 mg. E at 2½ hr.	Survived	13.5	F	Survived	Anesthetized
3	11.0	F	100 mg. Fh	10	None	0	11.0	F	4½	Anesthetized; treated animal died 5 min. after injection of endotoxin
4	7.0	F	100 mg. Fh	10	None	Survived	7.0	F	18	Anesthetized
5	10.0	M	100 mg. Fh	13	None	12	10.0	M	6	Anesthetized
6	8.0	F	100 mg. Fh 50 mg. E	5	50 mg. Fh at 1 and 2 hr.	22	11.0	F	10½	Anesthetized
7	7.0	M	100 mg. Fh 50 mg. E	9	50 mg. Fh at 2 hr.	2	9.5	M	6	Anesthetized
8	11.5	M	100 mg. Fh 100 mg. E	15	50 mg. Fh at 1 and 2 hr.	10	12.0	M	5	
9	11.0	F	100 mg. Fh 50 mg. E	18	50 mg. Fh at 1 and 2 hr. 50 mg. E at 4 hr.	Survived	12.0	F	5½	
10	13.0	M	100 mg. Fh 50 mg. E	23	50 mg. Fh at 1 and 2 hr. 50 mg. E at 4 hr.	12	10.5	M	12	
11	8.2	F	35 mg. △Fh	60	None	7½	7.8	F	5	
12	6.4	M	35 mg. △Fh	60	None	30	10.8	M	8½	

Key to abbreviations: I.M. = intramuscular injection; Fh = hydrocortisone hemisuccinate; △Fh = prednisolone hemisuccinate; E = cortisone acetate.

sex, and age of the treated and control dog were closely matched. Twelve treated and 12 simultaneous control animals were studied. In some experiments, supplementary doses of hydrocortisone hemisuccinate or cortisone acetate were given by the intramuscular route shortly before and at intervals after the administration of endotoxin (Table V). The same lot of "crude" *E. coli* endotoxin in a dose of 0.2 to 0.3 ml. per kilogram was used for all experiments. Seven pairs of dogs were anesthetized with pentobarbital and femoral artery pressure was recorded for 3 hours after injection of the endotoxin. In the remaining 5 experiments, the animals were not anesthetized so that any clinical differences could be observed. Rectal temperatures were studied in 2 pairs.

Characteristic hemodynamic and clinical responses were not clearly altered by pretreatment with the corticosteroids. Three of the treated animals but only one control animal survived (Table V). The average survival of the 11 control animals was only 7.5 hours. These data suggest that corticosteroids provided protection under the circumstances of these experiments but the differences are not decisive. Further studies with particular attention to the dose of endotoxin may lead to a clearer definition of the possible protection afforded by these agents in the dog.

DISCUSSION

The remarkable similarity of anaphylactic shock and shock that follows the injection of endotoxin suggests that a common pathway is involved in both reactions. A single injection of peptone, trypsin, hydatid cyst fluid, snake venom, the extract of *Ascaris lumbricoides*, polyvinylpyrrolidine (PVP), acacia, and fat emulsions also produce reactions which, though not identical, simulate anaphylaxis.[32, 58-64] Seventy-one different substances, including organic colloids such as starch, glycogen, and pancreatin, have produced the anaphylactic type of reaction; and also included are several inorganic chemicals such as arsenous sulfide and colloidal iron.[58]

"Anaphylactoid shock" has been a descriptive term that is widely used in indicating the close resemblance of the reaction to anaphylactic shock, although the basic mechanism involved in the anaphylactoid reaction is not clearly understood. Recent investigation has suggested that these reactions may be closely related to the molecular size of particles injected.[61] This would, in part, explain the diversity of chemically unrelated substances that produce this reaction. Peptones, carbohydrate polymers, fat emulsions, and colloidal solutions of certain inorganic materials are each composed of macromolecules of intermediate size. Purified endotoxins are also made up of macromolecules in the 100,000 to 1,000,000 range.[65]

The fundamental question of how macromolecules "trigger" anaphylaxis and anaphylactoid reactions in the dog still remains unexplained. The conclusion that the reactions are very closely related to changes in the liver and that they do not occur in the absence of the liver is well documented. Thomas and Essex[66, 67] have demonstrated that occlusive spasm of the intrahepatic vasculature accompanies anaphylactic and anaphylactoid shock and this conclusion is supported by studies from this laboratory.[12, 15, 16] Spasm of small hepatic veins explains the elevation of portal venous pressure, severe engorgement of the liver and intestine, and marked edema of the gall bladder. Shock results from the pooling of blood behind the small tributaries of hepatic veins with resulting

loss of large quantities of blood from the active circulation. This effect is most important in producing the profound hypotension that so rapidly follows injection of the shocking agent. The role of histamine in this sequence of events has been much debated but present evidence suggests that anaphylaxis and anaphylactoid shock are not the result of its action.[28, 48, 68]

In contrasting anaphylactic shock and the reaction to endotoxin in the dog, failure to obtain significant prolongation of the coagulation time with endotoxin has already been cited as an important point of difference. In addition, certain delayed effects of endotoxin may not be directly related to its anaphylactoid activity. One such delayed effect has been noted after hepatectomy. Whereas the initial response of shock following injection of endotoxin is prevented by preliminary hepatectomy, the lethal effect is augmented.[16] This is in contrast to the absence of any injurious effect when attempts are made to provoke anaphylaxis in a sensitized dog after the liver has been removed. Braude and associates[69] have shown that circulating endotoxin is, in fact, removed by the liver so that the absence of the liver may cause intensification of a delayed toxic effect which is apparently not related to its anaphylactoid activity. Studies are now in progress to define the nature and importance of this delayed effect more fully. These differences, however, do not minimize the essential similarity of the reactions produced by endotoxin, anaphylaxis, and anaphylactoid shock.

The occurrence of sudden shock in human patients with bacteremia caused by gram-negative microorganisms is probably related to the liberation of endotoxin from the bacteria. The clinical manifestations in severe cases may resemble anaphylaxis with sudden prostration, profound hypotension, bloody vomiting and bloody diarrhea, and a rapidly fatal course.[3, 5] Severe congestion of the liver, necrosis of liver cells, and submucosal hemorrhage, not unlike that observed in the experimental animals, are commonly found at autopsy.[70]

These studies admittedly leave the fundamental questions concerning pathogenesis unanswered. However, the concept that bacteremic shock is an anaphylactoid response permits a larger body of experimental and clinical knowledge to bear on this problem. Indeed, studies to date emphasize the need for further knowledge of the nature of the "trigger" which initiates the sequence of events in anaphylaxis or anaphylactoid shock.

SUMMARY AND CONCLUSIONS

When endotoxins from gram-negative microorganisms were injected intravenously into dogs, the animals became severely ill. The following series of events was observed: a precipitous fall in blood pressure, marked hyperpnea, excitement and gagging, apathy, and later coma. Massive evacuation of the bowels and vomiting occurred soon after the injection and this frequently led to profuse bloody vomitus and tenesmus with bloody diarrhea. Death followed within 3 to 18 hours and autopsy showed severe liver congestion, submucosal intestinal bleeding, and gall bladder edema.

The onset of shock was accompanied by a rise in portal vein pressure and the cause of shock was traced to massive pooling of blood in the portal system resulting in a greatly diminished venous return. An immediate marked fall in

leukocytes and platelets also followed the injection of endotoxin. The blood sugar was slightly elevated but subsequently fell to low levels after the second hour. Chordotomy, vagotomy, and decapitation did not alter the essential hemodynamic response and this indicated that shock was not primarily due to intoxication of the central nervous system. Histamine-like substance appeared in the blood of the inferior vena cava immediately after shock was produced. Pretreatment with adrenocortical hormones provided only questionable protection against the reaction. These studies demonstrated that shock produced by a single injection of endotoxin was, in each of these respects, simulated to those documented in the case of anaphylactic shock in the dog.

Two important points of difference were noted between shock caused by endotoxin and that due to anaphylaxis. No change in blood coagulability occurred after endotoxin, and this was in contrast to the liberation of heparin and prolongation of clotting time which characteristically occurs during anaphylaxis. Moreover, endotoxins appear to have an additional delayed toxic effect that leads to rapid death after hepatectomy.

The reaction to endotoxin is thought to be another example of the anaphylactoid type of reaction. Its close relationship to shock produced by a diversity of unrelated agents, including peptones, carbohydrate polymers, parasitic extracts, and snake venoms and the shock in patients with bacteremia which produce a similar response, have been discussed.

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TRANSMISSION OF THE EXPERIMENTAL COMMON COLD IN VOLUNTEERS

II. The Effect of Certain Host Factors Upon Susceptibility

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THE reasons why some persons have more colds than others are not known. The age of the subject[1-4] and the frequency and intimacy of exposure to persons with a common cold[5] have been shown to be important factors. Women acquire "natural" colds more frequently than men[1, 4, 6, 7] and more frequently develop the common cold after experimental inoculation.[8] Other unknown factors appear to influence the development of the common cold, however, since certain persons remain more susceptible to colds from year to year[2, 9, 10] and some persons are more susceptible to repeated challenges with infectious secretions than others.[8]

In the course of observations of the development of the common cold in adult volunteers following nasal instillation of infectious secretions, we attempted to study any additional factors which might be related to susceptibility to the common cold. We are presenting herewith our findings with regard to allergy, the presence or absence of tonsils and adenoids, the smoking of tobacco, and menstruation. Observations on the effect of chilling, as such, upon the development of the experimentally transmitted common cold are being reported separately.[11]

MATERIALS AND METHODS

Secretions obtained from patients with typical common colds were filtered through an ultrafine sintered glass filter. After bacteria were shown to be absent by culture and there was no demonstrable effect upon embryonated eggs, suckling mice, adult mice, or on HeLa cells in tissue culture, the secretions were diluted 1:10 and stored in a 5 per cent solution of hemoglobin, obtained from group O cells buffered to a pH of 7.4 at −70° C. Immediately preceding instillation the solution was again diluted 1:10 with 0.15 M phosphate buffered saline solution, pH 7.4. The control for these secretions was a solution of hemoglobin obtained from group O cells, buffered in the same way. Inoculations were made by instilling 0.2 ml. of the solution into each nostril.

After these laboratory studies were performed on each secretion, it was instilled into the nares of 6 to 10 human volunteers to determine whether it would cause symptoms of the common cold. All volunteers were healthy men or women between the ages of 18 and 35 years.

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Special rooms or chambers were used to control the temperature and humidity of the environment. Men remained for 2 to 4 hours in the specified environment, dressed in shorts and socks; women in shorts, socks, and halters or brassières. A chilling temperature of 60° F. with an 80 per cent relative humidity for 4 hours and a comfortable temperature of 80° F. with a 30 per cent relative humidity were selected for the experiments. The infectious secretions or control solutions were instilled intranasally immediately prior to chilling or at the end of the period of chilling.

For experiments at lower temperatures (10° F. and 80 per cent relative humidity for 2 hours) the subjects were dressed in street clothes plus overcoat, hat, and gloves. At the end of the period in the special-temperature rooms, the intranasal instillations were made and the subjects were dismissed. They reported for examinations on the second, third, fourth, and sixth days after the experiment. Initial examinations of the nose and throat were made and a history of respiratory symptoms was obtained at the beginning and end of each experiment and on each of the subsequent visits. These examinations, and the subsequent decision as to whether or not a cold was present, were made by members of the research team who were unaware of the experimental group into which the patient had been placed.

The criteria for identifying the development of a cold in a volunteer and the reasons for their adoption will be given in detail in another publication.[12] In brief, in order to be classified as having developed a cold in connection with an experiment, the subject had to meet 2 of the following 3 criteria:

1. A score of 14 or more points in 6 days (over the base-line symptoms taken on the day of the experiment) based on the following symptoms recorded daily: headache, sneezing, chilliness, nasal discharge, nasal obstruction, sore throat, coughing, and malaise.

2. The impression of the subject that a cold had developed following challenge.

3. An increase in nasal discharge on 3 of the 6 days after challenge (or severe nasal discharge on 2 days).

RESULTS

History of Allergy.—In Table I is shown the frequency with which colds developed in allergic and nonallergic persons. A history of allergy was given by 143 of the subjects who were challenged with one of 5 infectious secretions obtained from patients with the common cold. Colds developed in 64 or 45 per cent of these subjects. These results are to be compared with 218 (31 per cent) colds that developed in 693 persons who also received one of the same 5 infectious secretions but gave no history of allergy.

Among subjects who gave a history of allergic symptoms following the inhalation of certain substances, colds developed more frequently (48 per cent) than among subjects whose allergic symptoms followed the injection or ingestion of particular substances (40 per cent). The statistical possibility that the differences between these 2 groups and the nonallergic group occurred by chance was less than 1 in 100.

The differences among the male and the female subjects showed the same trend as in the entire group. However, the number of observations in the female groups were insufficient to give statistical significance to the differences observed.

Lines 1 to 4 in Table I represent subjects who received no infectious secretions and whose upper respiratory infections, therefore, represented spontaneous "colds." Spontaneous infections were more frequent in subjects with

TABLE I. FREQUENCY OF COLDS IN RELATION TO A HISTORY OF ALLERGY

LINE NO.	RECEIVED INFECTIOUS SECRETION	TYPE OF ALLERGY	MEN			WOMEN			BOTH SEXES		
			NO. OF SUBJECTS	DEVELOPED COLD		NO. OF SUBJECTS	DEVELOPED COLD		NO. OF SUBJECTS	DEVELOPED COLD	
				NO.	PER CENT		NO.	PER CENT		NO.	PER CENT
1	No	None	125	13	10	126	13	10	251	26	10
2		Inj.-Ing.*	10	1	--	14	2	--	24	3	13
3		Inhalant	18	2	--	16	3	--	34	5	15
4		All allergic	28	3	11	30	5	17	58	8	14
5	Yes	None	380	101	27	313	117	37	693	218	31
6		Inj.-Ing.*	33	12	36	22	10	46	55	22	40
7		Inhalant	69	33	48	19	9	47	88	42	48
8		All allergic	102	45	44	41	19	46	143	64	45
Lines 1 vs. 2 vs. 3			Not significant $X^2 = 13.07$; $P = 0.01$			Not significant $X^2 = 1.24$; $P = 0.1$			Not significant $X^2 = 10.04$; $P = 0.01$		
Lines 5 vs. 6 vs. 7											

*Allergic reactions from substances injected or ingested.

TABLE II. FREQUENCY OF COLDS IN RELATION TO HISTORY OF SMOKING TOBACCO OR OF TONSILLECTOMY

RECEIVED INFECTIOUS SECRETION	HISTORY	MEN			WOMEN			BOTH SEXES		
		NO. OF SUBJECTS	DEVELOPED COLD		NO. OF SUBJECTS	DEVELOPED COLD		NO. OF SUBJECTS	DEVELOPED COLD	
			NO.	PER CENT		NO.	PER CENT		NO.	PER CENT
No	Tonsillectomy	66	6	9	81	7	9	147	13	9
	No tonsillectomy	89	12	14	73	10	14	162	22	14
Yes	Tonsillectomy	209	66	32	154	56	36	363	122	34
	No tonsillectomy	265	68	26	199	79	40	464	147	32
No	Tobacco smoking	34	5	15	44	6	14	78	11	14
	No tobacco smoking	32	4	13	72	7	9	111	11	10
Yes	Tobacco smoking	130	47	36	119	41	34	249	88	35
	No tobacco smoking	156	43	28	172	69	40	328	112	34

inhalant allergy (15 per cent) than in those with allergy to injected or ingested substances (13 per cent) which in turn were more frequent than in subjects with no history of allergy (10 per cent). These differences, however, were not statistically significant.

Presence or Absence of Tonsils and Adenoids.—Subjects were questioned as to whether their tonsils and adenoids had ever been removed. The answers were verified by an examination of the nose and throat. The difference in the number of colds that developed in the 2 groups was negligible. (Table II.)

Among the subjects who received infectious secretions, and whose tonsils had previously been removed, 122 of 363 (34 per cent) developed colds; whereas 147 of 464 (32 per cent) of subjects who gave no history of tonsillectomy developed colds. Among the subjects who did not receive infectious secretions and whose tonsils had previously been removed, 13 of 147, or 9 per cent, had symptoms of a cold, as compared with 22 of 162, or 14 per cent, of subjects who still retained their tonsils. The differences between tonsillectomized and nontonsillectomized subjects were not statistically significant in either the infected or the noninfected group.

Tobacco Smoking.—Information regarding smoking habits was available in 328 subjects who received infectious secretions and in 111 subjects who received control solutions intranasally. The subjects stated whether or not they were smokers at the time of the experiment, but in practically every instance those who were not smoking at the time of the experiment had never smoked at any time. All were cigarette smokers, although some occasionally smoked a pipe also. There was no significant difference in the frequency of experimental colds in the two groups. (Table II.)

Among the subjects who received infectious secretions, 35 per cent of the smokers and 34 per cent of the nonsmokers developed colds. Among those who received control solutions, 14 per cent of the smokers and 10 per cent of the nonsmokers had symptoms of a cold. The differences between the smokers and nonsmokers were not statistically significant in either group.

Time in Menstrual Cycle When Experiment Was Performed.—Information with regard to menstruation is available on 54 female subjects who were chilled during an experiment and 232 who were not subjected to chilling. Each subject was asked the date of the onset of her last menstrual period. Susceptibility appeared greater during the intermenstrual period and perhaps related to ovulation. Accordingly, the data were tabulated upon the assumption that ovulation occurred at the mid-point of the menstrual cycle, which was assumed to be fourteen days from the beginning of the menstrual period that immediately preceded the experiment. In Figs. 1 and 2, and Table III, the frequency with which colds developed in these subjects is shown in relation to the time before or after the mid-point of the menstrual cycle at which the experiment was performed (i.e., the infectious challenge or control solution was given).

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Among the subjects who were not chilled, and who were challenged in the interval between the onset of the menstrual period and the fifth day before the mid-point of the menstrual cycle (that is, in the menstrual and immediately postmenstrual phase of the cycle) 23 per cent developed colds. When the challenge was given during the mid-portion, or the ovulatory phase, of the menstrual cycle, 37 per cent of the subjects developed colds. An infectious challenge given during the last or premenstrual part of the cycle caused the highest percentage of colds (41 per cent). These differences were not considered significant, except possibly in one instance. The probability that the difference between Lines 5 and 7 occurred by chance was less than 1 in 25.

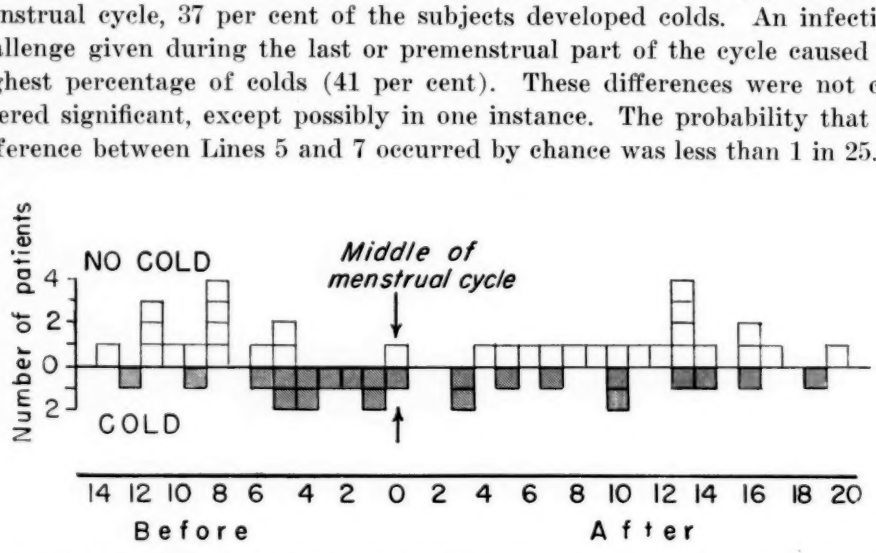

Fig. 1.—Number of experimental colds in relation to the mid-point in the menstrual cycle in subjects who were chilled.

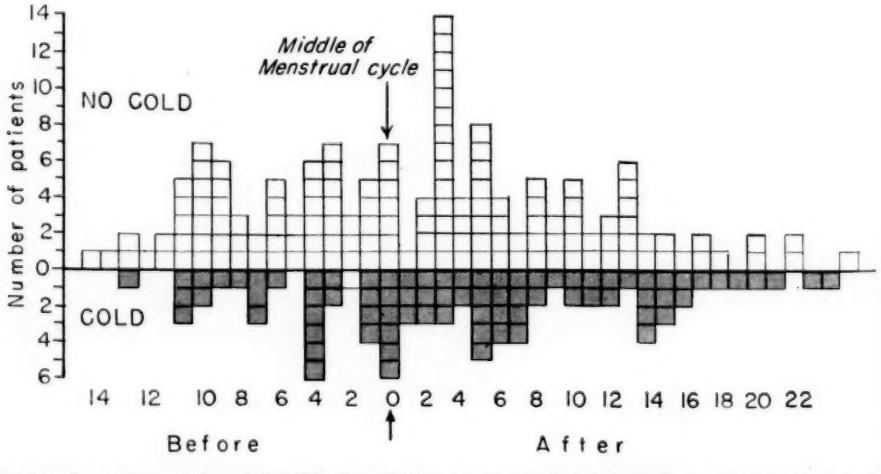

Fig. 2.—Number of experimental colds in relation to the mid-point in the menstrual cycle in subjects who were not chilled.

Among the chilled subjects, colds developed much more frequently in subjects challenged during the ovluatory phase, which was assumed to be from 4 days before to 5 days after the mid-point of the menstrual cycle (10 colds in 13 subjects) as compared with subjects challenged earlier in the menstrual

cycle (5 colds in 18 subjects) or those challenged later in the menstrual cycle (7 colds in 23 subjects). The probability that these differences occurred by chance was less than 1 in 100.

When the incidence of colds among all the chilled patients was compared with that among the patients who were not chilled during the experiment the difference was not significant. When the 2 groups of subjects who were challenged during the ovulatory phase of the menstrual cycle were compared, the incidence of colds was much greater among the chilled subjects (10 colds in 13 subjects) than among subjects who were not chilled (36 colds in 97 subjects). The probability that this occurred by chance was less than 1 in 100.

TABLE III. FREQUENCY WITH WHICH EXPERIMENTAL COLDS DEVELOPED IN WOMEN: RELATIONSHIP OF MID-POINT IN MENSTRUAL CYCLE TO THE DAY ON WHICH THE EXPERIMENT WAS CONDUCTED

LINE NO.	SUBJECT TO CHILLING	DAY OF EXPERIMENT (NUMBER OF DAYS BEFORE OR AFTER MID-POINT OF MENSTRUAL CYCLE)	NUMBER OF SUBJECTS		
			CHALLENGED WITH INFECTIOUS SECRETION	DEVELOPED COLDS	
				NO.	PER CENT
1	Yes	5 days or more before	18	5	28
2	Yes	4 days before to 5 days after	13	10	77
3	Yes	6 days or more after	23	7	30
4	Yes	Total chilled	54	22	41
5	No	5 days or more before	52	12	23
6	No	4 days before to 5 days after	97	36	37
7	No	6 days or more after	83	34	41
8	No	Total unchilled	232	82	35

	X^2	P		X^2	P
Line 1 vs. line 2	7.30	0.01	Line 3 vs. line 7	0.84	N.S.
Line 2 vs. line 3	7.20	0.01	Line 4 vs. line 8	0.55	N.S.
Line 2 vs. line 6	7.47	0.01	Line 5 vs. line 6	3.05	N.S.
Line 2 vs. line 5	13.47	0.001	Line 5 vs. line 7	4.55	0.04
Line 2 vs. line 7	5.85	0.02			

DISCUSSION

Data obtained from clinical histories, epidemiologic investigations, and experimental infections[13, 14] all indicate that some individuals develop colds more frequently than others. It is extremely important to delineate the host factors that are related to susceptibility to the experimental common cold in order to find the most favorable situations under which the experimental cold may be produced and, if possible, to find the nature of the resistance to infection.

We have found that tobacco smoking and the prior removal of tonsils bear no relationship to the frequency of development of the experimental common cold. Buck[4] reported that tobacco smoking had no appreciable effect on the incidence of naturally acquired colds in a group of 45 families.

With regard to tonsillectomy, our observations are in accord with the conclusions of most observers who studied the relationship of previous tonsillectomy to the development of naturally acquired colds.[15-19] The decrease in

the frequency of the common cold which follows a tonsillectomy in some children can be accounted for by one or both of the following facts. First, the tonsils are often removed because of an unusually high frequency of upper respiratory infections in the years immediately preceding the operation, whereas these infections may reach a frequency that is nearer the average after or within a year or two after the tonsillectomy. Second, since each child is older after the tonsillectomy than before, the frequency of colds is likely to be less.

The statement is often made that allergy is predisposing to colds. Siegel and his associates[20] stated that among a group of adults who reported 5 or more colds a year 19.3 per cent gave a history of asthma, hay fever, allergic rhinitis, or eczema. Among those who reported less than 3 colds a year, only 10.1 per cent gave a similar history. A family history of allergy and eosinophilia of the nasal secretions were also more frequent among the subjects who reported 5 or more colds a year. Our experiments demonstrated a slight but significant increase in susceptibility to the common cold in subjects who gave a history of allergy. It might be contended that the solutions that were instilled in the subjects' nostrils contained substances to which certain subjects were allergic. We doubt the validity of this explanation since the solutions were nasal secretions that had been rendered cell-free and for the most part bacteria-free and to which only hemoglobin obtained from group O cells had been added. Furthermore, we believe that the rigid criteria that were applied for the diagnosis of a cold would eliminate a mild allergic rhinitis, whereas the observers and the allergic subjects themselves would have diagnosed a more severe allergic rhinitis had it been present. At any rate, whatever the mechanism, it is obvious that more persons with a history of allergy have coryzal symptoms of significance after exposure to an infectious agent than persons without allergy.

The increased incidence of colds among subjects who were challenged with infectious secretion and subjected to chilling during the mid-portion of the menstrual cycle is an extremely interesting observation. We know of no information on this subject with regard to the common cold, but Weinstein and his associates[21] studied the relationship of the time during the menstrual cycle when the clinical manifestations of poliomyelitis appeared. Among 50 women, the menstrual period began in the 5 days before the first clinical manifestation of poliomyelitis in 30 per cent and in the 4 days after the onset of the disease in 48 per cent. In 14 per cent of the patients the menstrual period began from the fourteenth to the sixth day before the disease and in only 8 per cent the menstrual period started somewhere between the fifth and thirteenth day after the onset of the disease. This relationship was not considered to be the result of a change in the length of the intermenstrual interval, since none of the patients gave a history of an early or late onset of menstruation for that particular month. The authors concluded that "there may be a critical point in the menstrual cycle in respect to susceptibility to clinical poliomyelitis at an interval corresponding to the incubation period preceding the beginning of menstruation or about 12 days prior to the onset of menstrual flow."

We have recalculated these data to determine the relationship of the beginning of a 12-day incubation period to the mid-point of the menstrual cycle (assuming a 28-day menstrual cycle); as shown in Fig. 3, the presumed beginning of the incubation period fell between the 4th day before and the 5th day after the mid-point of the menstrual cycle in 39 (78 per cent) of the 50 cases. This is the same period in which we found the greatest frequency of development of experimental colds.

Why subjects who are challenged and subjected to chilling around the middle of the menstrual cycle are more likely to develop a common cold than subjects who are challenged at that time without being chilled or than subjects who are challenged and chilled during another part of the menstrual

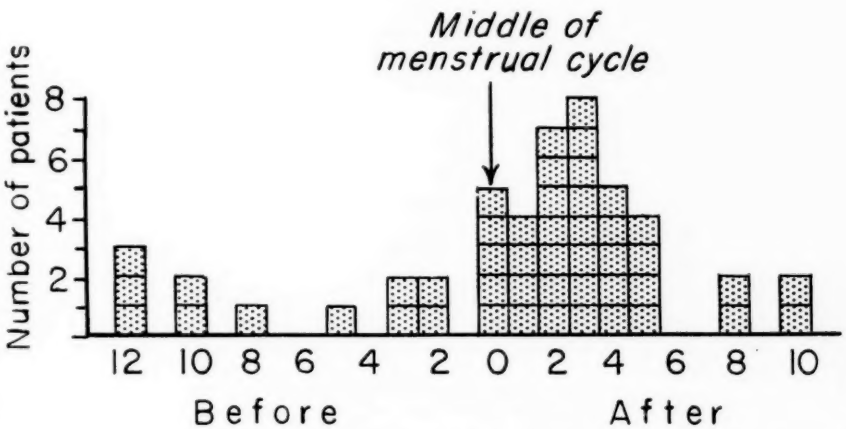

Fig. 3.—Onset of cases of poliomyelitis in relation to the mid-point in the menstrual cycle. (Recalculated from Weinstein and associates.[21])

cycle is a matter for speculation. It has long been noted that engorgement of the nasal mucosa occurred during the premenstrual and menstrual periods and during pregnancy.[22, 23] Amberson[24] observed congestion of the bronchial mucosa around the time of the menstrual period. Mortimer and his associates[23] found that the injection of estrogens in monkeys could produce turgescence of nasal mucous membranes. On the other hand, Inouye, Jackson, Dowling, and Spiesman[25] were unable to establish any correlation between the appearance of the mucous membranes of the nose at the time of challenge with an infectious secretion and the subsequent development of a common cold. It is possible that estrogen or some other endocrine secretion acts upon the general metabolism or in some other way to produce resistance to the common cold.

SUMMARY

1. Among 143 allergic subjects between the ages of 18 and 35 years who were challenged with infectious secretions taken from patients with a common cold, 45 per cent developed colds, as compared with 31 per cent among 693 nonallergic subjects who were challenged in the same way. These differences were statistically significant.

2. Among 363 subjects whose tonsils had previously been removed, colds followed experimental challenge in 34 per cent, as compared with 32 per cent colds among 464 subjects whose tonsils were intact.

3. Among 249 persons who smoked tobacco, 35 per cent developed colds following experimental challenge, as compared with 34 per cent among nonsmokers.

4. Among 13 female volunteers who were chilled and given an infectious secretion during the middle third of the menstrual cycle, 10 (77 per cent) developed colds as compared with 28 and 30 per cent of subjects similarly treated who were challenged during the first or the last third of the menstrual cycle, respectively. The differences between the incidence of colds in chilled subjects challenged during the middle third and those chilled and challenged during the first or last thirds of the menstrual cycle or those challenged during the middle third of the menstrual cycle without being chilled, were statistically significant.

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STUDIES ON THE ROUTINE LABORATORY DIAGNOSIS OF COXSACKIE GROUP B VIRUS INFECTIONS

I. The Application of Tissue Culture Procedures

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VIRUSES of the Coxsackie B group have been isolated from cases of pleurodynia, aseptic meningitis, suspected or proved poliomyelitis, nondifferentiated febrile illnesses, and even apparently healthy individuals.[1] While in some of the cases an etiologic relationship could be clearly established, in others the presence of the virus might have been coincidental. In any event, it is obvious that a virus diagnostic laboratory must have convenient routine procedures for isolation and identification of these agents and for detection of antibody responses in the patients.

While the use of suckling mice led to the discovery of the Coxsackie B viruses, it does not lend itself readily to large-scale testing such as a virus diagnostic laboratory may be called upon to perform. Identification of isolated agents by serologic typing and serum neutralization tests with the patients' sera would require too large a number of animals. Crowell and Syverton[2] were the first to report on adaptation of B_1, B_3, and B_4 viruses to tissue cultures of HeLa cells but they experienced difficulties in cultivating B_2. A new member of the group, B_5, was isolated in tissue culture by Steigman.[3] Sickles and associates[4, 5] reported on the use of tissue cultures of HeLa or monkey renal cells for isolation of certain Coxsackie A and Coxsackie B viruses.

This report deals with the development of techniques, their application to routine diagnosis of sporadic cases of Coxsackie B infections, and comparisons in a few instances of the results recorded in tissue culture with those obtained in suckling mice.

MATERIALS AND METHODS

Tissue Culture Techniques.—The HeLa strain of human cancer cells was employed in these studies. Initially it was maintained as a laboratory stock culture as described by Scherer and associates,[6] and the growth medium used was composed of 40 per cent human serum, 58 per cent Hanks' balanced salt solution (BSS), and 2 per cent acetone-extracted chick embryo extract. Later the growth medium used was 20 per cent human serum and 80 per cent Basal Medium (Eagle) in Hanks' BSS.[7] Before test tube culture production, the sheets of cells in the stock culture flasks were washed with 3 changes of 10 ml. of Hanks' BSS and the cells were then suspended by trypsinization or by scraping with a rubber policeman into a medium which was composed of 10 per cent horse serum, 60 per cent maintenance solution of Scherer,[8] and 30 per cent tissue culture medium No. 199. More

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recently the medium employed has been 10 per cent horse serum and 90 per cent Basal Medium (Eagle) in Hanks' BSS. The tube cultures were seeded with 0.6 ml. of the cell suspension containing approximately 6×10^4 cells. After two days this growth medium in the tubes was replaced with 1.0 ml. of maintenance medium containing, during earlier experiments, 10 per cent horse serum, 30 per cent No. 199 and 60 per cent Earle's BSS reinforced with a small amount of casein hydrolysate* (final concentration 1:500). For later experiments 10 per cent horse serum and 90 per cent Basic Medium Eagle in Earle's BSS were employed. The Earle's BSS was substituted for the Hanks' BSS in the tube maintenance medium because the higher buffering capacity of the Earle's solution allowed longer incubation without fluid renewal.

Viruses.—

Laboratory strains: The B_1 (Connecticut 5) and the B_4 (Powers) strains were obtained through the courtesy of Dr. R. S. Huebner of the National Institutes of Health; the B_2 (Redmond) and B_3 (Nancy) strains from the American Type Culture Collection; and the B_5 strain from Dr. G. Dalldorf in the fifth mouse passage. All strains were subjected to 2 to 6 further passages in suckling mice in this laboratory prior to adaptation to tissue culture.

Clinical specimens: Stools were received by the virus diagnostic laboratory from patients with suspected Coxsackie or poliomyelitis infections. Twenty per cent suspensions were prepared in sterile distilled water and clarified by centrifugation at 2,500 r.p.m. for 20 minutes. The supernatant fluids were treated with penicillin and streptomycin (1,000 units and 1.0 mg. per milliliter, respectively) and incubated at 36° C. for 60 minutes prior to inoculation into culture tubes. Where mold contamination was found, mycostatin (Squibb) was employed in final concentration of 1,000 units per milliliter of stool suspension. Later the stool specimen was swabbed with a cotton swab which was placed into 2.0 ml. of Hanks' BSS containing the above-mentioned amounts of penicillin, streptomycin, and mycostatin. The swab was incubated in the Hanks' solution for 30 minutes at 37° C., excess fluid was drained, and the swab was discarded. Numerous comparisons between the former and the latter method of preparation of stool specimens showed a good correlation. Since the stool swab method was technically the easier and speedier one, it was finally selected as the routine method.

Rectal and throat swabs: These were placed into 2.0 ml. of Hanks' BSS and frozen at the time of collection. The fluid was expressed from the swab after thawing and the swab was discarded. The preparation was lightly centrifuged (when necessary) prior to treatment with penicillin, streptomycin, and mycostatin as described above.

Spinal fluids: The fluids, collected aseptically and frozen until used, were not subjected to incubation with antibiotics.

All specimens were inoculated into 3 culture tubes each, employing 0.2 ml. volumes; or intraperitoneally into one litter of suckling mice each, using 0.03 ml. per animal.

Immunologic Typing of Isolated Viruses.—The media from cultures showing cellular degeneration were harvested and routinely diluted to 10^{-2} after it was found, on numerous titrations of such fluids, that the virus titers were approximately 10^{-4} to 10^{-5}. The diluted fluid, 0.3 ml. volume, was added to each of 4 tubes containing 0.3 ml. of appropriately diluted hyperimmune sera, respectively: (1) Polio-Coxsackie B mixture, a pool containing poliovirus types I to III antisera and Coxsackie group B types 1 to 5 antisera; (2) Polio-mixture, a pool of poliovirus types I to III antisera; (3) Coxsackie B mixture, a pool of Coxsackie B types 1 to 5 antisera; and (4) Hanks' BSS solution.

After one hour at room temperature, culture tubes were inoculated with 0.2 ml. of one of the various sera-virus mixtures and incubated at 36° C. until tissue degeneration was seen in the control, usually 24 to 48 hours after inoculation. After it was established whether the isolate belonged to the poliomyelitis group or Coxsackie B group of viruses, the virus type was determined, using type-specific sera following the above procedure.

Virus Titrations in Mice.—Serial tenfold dilutions were prepared in phosphate buffered saline and one litter of 1- or 2-day suckling mice (8 mice per litter) was inoculated

*Parenamine; Winthrop-Stearns, Inc., New York, N. Y.

per dilution employed. Each animal received 0.03 ml. of the appropriate dilution by the intraperitoneal route. They were observed twice daily for a period of 2 weeks for typical paralysis and death.

Virus Titrations in Tissue Culture.—These were performed in duplicate cultures of HeLa cells employing as inoculum 0.2 ml. of virus in serial tenfold dilutions in Hanks' BSS. Cultures were observed daily for at least 7 days.

Sera.—Hyperimmune sera were prepared in 5- to 6-week-old Syrian hamsters employing hamster-adapted Coxsackie B virus strains. For immunization, 0.2 ml. of a 20 per cent suspension of infected suckling hamster muscle was inoculated intraperitoneally. Three injections were given at 6-day intervals and the animals were bled by cardiac puncture one week after the third inoculation.

Patients' sera were prepared from bloods collected as near to the time of onset of illness as possible and 2 to 3 weeks later. These were inactivated at 56° C. for 30 minutes and stored at −20° C. until serum neutralization tests were performed.

Serum Neutralization Tests.—Hyperimmune animal sera: Virus and serum dilutions were prepared in Hanks' BSS (containing ⅓ the usual amount of $NaHCO_3$) and 0.3 ml. of a virus dilution was added to 0.3 ml. of a serum dilution. Following incubation of the mixtures for one hour at room temperature, 2 tissue culture tubes were inoculated with 0.2 ml. each and incubated at 36° C. Cultures were examined for 8 days before terminating the experiment. Suckling mice were inoculated with 0.03 ml. of the serum-virus mixtures, one litter per preparation, and observed for a period of 2 weeks.

Each test was accompanied by virus titrations. The viruses were initially titrated in the presence and absence of normal hamster serum. However, since no differences in titers were noted, Hanks' BSS was employed in later control titrations.

Patients' sera were diluted in twofold steps in Hanks' BSS starting at a 1:10 or 1:16 dilution, and 0.3 ml. volumes were distributed into 5 sets of tubes. Tissue culture fluids of the 5 prototype viruses, diluted to contain 100 tissue culture doses per 0.3 ml. (based on a 2-day cytopathogenic end point), were then added to the serum dilutions; sets 1 to 5, respectively. Mixtures containing 0.3 ml. of the initial serum dilution and 0.3 ml. of Hanks' BSS served as a serum control for each specimen. Incubation, inoculation, and virus titrations were carried out as outlined above. The results were recorded after the control virus titrations indicated that 100 tissue culture doses were being employed as virus challenge (48 hours).

When suckling mice were employed for the neutralization tests with patients' sera, 100 LD_{50} of mouse-adapted virus were employed, and the animals observed for a period of 2 weeks.

RESULTS

The Development of Standard Virus and Antisera.—Preliminary investigation dealt with adaptation of the 5 type B strains to growth in HeLa and with the specificity of hyperimmune sera in tissue culture neutralization tests.

1. *Adaptation of type B Coxsackie strains to growth in cultures of strain HeLa:* Passages in vitro were performed by inoculating 0.1 ml. of a dilution of infectious material (prepared in Hanks' BSS) into culture tubes containing 1.0 ml. of the maintenance fluid. On first passage, the 5 prototype strains produced definite cytopathogenic effects even in dilutions of $10^{-3.0}$ or greater (Column 2 of Table I). However, for rapid serial passages at 1- or 2-day intervals, lower dilutions were employed through the first few passages. Later passages were performed close to the limiting infective dilutions. After 6 passages in vitro the original infected mouse tissue had been diluted beyond the point where residual virus or toxicity of mouse tissue could be considered as the cause of degeneration in the cultures. These dilutions are listed in Column 5 of Table I. The differences in the final dilution factors are due to passage of

the 5 strains at varying dilutions. Furthermore, as will be shown below, the cytopathogenic changes were prevented by specific hyperimmune sera, establishing the specificity of the cellular degeneration.

Comparative titrations were performed in suckling mice and in HeLa culture tubes with both the starting seed (infected mouse tissue) and the culture fluids after 6 passages of the virus in vitro. The results are listed in Table I, Columns 1 to 4. The tissue culture titration results include the day when definite degeneration was first observed in tubes *at the titration end point.*

TABLE I. THE RESULTS OF TITRATIONS IN SUCKLING MICE AND TISSUE CULTURE (HELA) OF COXSACKIE B VIRUS STRAINS BEFORE AND AFTER PASSAGE IN VITRO

VIRUS STRAINS	ORIGINAL MOUSE VIRUS		TISSUE CULTURE PASSAGE NO. 6		DILUTION OF ORIGINAL VIRUS BY PASSAGE IN VITRO
	MOUSE LD_{50}/0.03 ML. 1	TCD*/0.2 ML. 2	MOUSE LD_{50}/0.03 ML. 3	TCD/0.2 ML. 4	5
B_1	$10^{6.5}$	$10^{5.0}$ 4-5 days†	$10^{6.5}$	$10^{7.0}$ 4 days†	10^{-22}
B_2	$10^{5.5}$	$10^{4.0}$ 8 days	$10^{3.2}$	$10^{4.0}$ 5 days	10^{-16}
B_3	$10^{6.0}$	$10^{5.0}$ 4-5 days	$10^{5.0}$	$10^{6.0}$ 3 days	10^{-20}
B_4	$10^{6.5}$	$10^{3.0}$ 6 days	$10^{5.0}$	$10^{3.5}$ 3 days	10^{-13}
B_5	$10^{6.5}$	$10^{4.5}$ 9 days	$10^{5.2}$	$10^{5.5}$ 6 days	10^{-10}

*TCD = tissue culture doses.
†Days of incubation when degeneration was first noted at end point dilution.

The infected mouse tissues had higher titers in mice than in tissue cultures for all 5 group B types, but on passage in vitro the mouse titers decreased for all but type B_1. Simultaneously, there was an increase in the tissue culture titers of all but types B_2 and B_4 which remained about the same. The titrations after 6 passages in HeLa cells indicated higher titers in vitro than in mice for all but type B_4 virus. Furthermore, after passage in vitro there was a marked reduction in the time necessary for tissue degeneration to be manifest. Five additional passages in HeLa cells did not increase the tissue culture titers. No mouse titrations were performed with the eleventh passage materials.

Storage of the 5 virus strains in the dry ice chest for one year as undiluted tissue culture fluid ($T.C._{-6}$) has resulted in a decrease in the tissue culture titers of less than 1 log.

TABLE II. HYPERIMMUNE SERUM TITRATIONS IN TISSUE CULTURE NEUTRALIZATION TESTS WITH COXSACKIE B VIRUS STRAINS

SERUM DILUTION	TISSUE CULTURE DOSES OF VIRUS STRAINS NEUTRALIZED				
	TYPE B_1*	TYPE B_2*	TYPE B_3*	TYPE B_4*	TYPE B_5*
Homologous†					
1:4	> 100,000	Not done	Not done	10,000	100
1:16	10,000	10,000	1,000	100	0
1:64	4,000	1,000	100	32	0
1:128	1,000	100	32	10	0

*Virus strains employed after 6 passages in cultures of strain HeLa.
†No heterologous neutralization was noted except a 1:4, but not a 1:10, dilution of B_1 serum inhibited 1 to 10 TCD of B_4 virus.

Quantitative neutralization tests with hyperimmune sera: For determining the titers and specificity of the hyperimmune sera prepared in hamsters, as well as of the tissue culture strains, "checkerboard" titrations were performed; i.e., several dilutions of the homologous and heterologous sera were

tested against several virus dilutions. Separate experiments were performed with each virus strain. Table II shows the results of homologous serum titrations. The 5 prototype sera proved to be highly specific even when heterologous serum dilutions of 1:2 or 1:4 were employed. Heterologous reactions were noted only in one instance where a 1:4, but not a 1:10, dilution of B_1 serum neutralized between 1 and 10 $T.C._6$ doses of B_4 virus.

The specificity noted permitted the use of these sera for immunologic typing of isolated agents, and for routine use as standard control sera in neutralization tests. A 1:20 dilution was selected for immunologic typing except for the B_5 serum where a 1:4 dilution was employed. Immunization with this type has given consistently low serum titers in both hamsters and guinea pigs, even after a rigorous immunization schedule.

The Use of HeLa Cells in the Diagnosis of Coxsackie Infections.—The availability of strain-specific hyperimmune sera and tissue culture strains of the Coxsackie B viruses made it feasible to apply the tissue culture techniques in routine diagnosis by: (1) isolation and subsequent identification of virus from clinical specimens; and (2) identification of specific antibodies in sera of patients.

TABLE III. COXSACKIE B VIRUS ISOLATIONS FROM VARIOUS TYPES OF CLINICAL SPECIMENS IN CULTURES OF STRAIN HELA

TYPE OF SPECIMEN	NUMBER OF STRAINS ISOLATED					TOTALS FOR EACH KIND OF SPECIMEN
	B_1	B_2	B_3	B_4	B_5	
Stool	1	11	24	13	4	53
Rectal swab	0	4	3	3	0	10
Throat swab	0	6	1	10	1	18
Spinal fluid	1	0	1	0	0	2
Totals for each virus type	2	21	29	26	5	83

1. *Isolation and immunologic typing of Coxsackie B virus strains:* Tube cultures of HeLa cells employed with a variety of clinical specimens resulted in 83 isolations representing all 5 types of Coxsackie B virus (Table III). Degeneration was noted in culture tubes from 1 to 7 days following inoculation and the culture fluids were harvested and passed in tissue culture in the presence of standard hyperimmune sera for the 3 immunologic types of poliomyelitis virus and the 5 immunologic types of Coxsackie B virus, as described above under Immunologic Typing of Isolated Viruses.

Successful isolation of strains representative of 4 immunologic types of Coxsackie B virus was noted from stools, rectal or throat swabs, and from 2 spinal fluids. In one case, 2 virus types, B_2 and B_4, were isolated and in this patient neutralizing antibodies developed to B_4 virus (1:64) but were not detected to B_2 virus ($< 1:16$). The quantity of serum was not sufficient to employ a lower serum dilution.

Parallel attempts at virus isolation in suckling mice and HeLa cultures were made in a few instances. While the evaluation of comparative susceptibility of the two systems must await collection of more extensive data, it is

apparent from the results presented in Table IV that primary isolation failed in tissue culture in some cases where the specimens yielded virus in suckling mice, and vice versa. The strains isolated in mice could be typed, however, directly in tissue culture without prior passage in HeLa cells. Likewise, those strains isolated in HeLa could be passed into mice directly after isolation in tissue culture. Thus, the differential results in the two systems may represent a problem of sampling or viral concentration since the isolation in mice of Coxsackie B strains which do not readily propagate in HeLa cultures has not presented a problem to date.

TABLE IV. COMPARATIVE ISOLATIONS OF COXSACKIE B VIRUS IN SUCKLING MICE AND IN CULTURES OF STRAIN HELA

		ISOLATION RESULTS IN MICE	
		+	−
Isolation results in HeLa	+	16	5
	−	11	101

2. *Identification of specific antibodies in sera of patients:* Results of serum neutralization tests in HeLa cell cultures with paired patients' sera are presented in Table V.

Groups A1 and A2: There was a fourfold or greater increase in antibody titer to the type of virus isolated in 20 of the 25 cases studied. Five of the 20 cases showed an heterologous response as well (Group A2). Two of these 5 cases revealed heterologous titer rises to the B_1 virus strain; another 2 patients showed high heterologous titers to the B_1 virus in both acute and convalescent sera without showing a rise to this agent; and one patient demonstrated an heterologous titer to B_4 virus.

Group B: In 2 of the 25 patients a specific increase of less than fourfold was noted. From one of these patients both B_2 and B_4 viruses were isolated with an antibody response resulting to the B_4 virus only.

Group C: Two other patients had high titers against the homotypic virus in both acute and convalescent sera.

Group D: Finally, in one case, only a slight increase in titer to the homotypic virus was noted while marked increases (sixteen fold or greater) were found for two other virus types.

Although the comparative serum neutralization titrations in mice were very limited, essentially similar results were obtained with the first three pairs of sera listed in Table V. That is, significant rises in serum titer were observed only against the homotypic type B_2 agent. These titers were $< 10/40$, $20/320$, $< 20/40$, respectively. In two of these cases a slight serologic response ($< 20/20$) was evidenced against the type B_1 agent as well. Since these few neutralization tests required more than 90 litters of mice, no further comparisons were made.

DISCUSSION

The 5 immunologic types of mouse-adapted Coxsackie B virus, including several strains of type B_2, were found to propagate readily in cultures of HeLa

TABLE V. COXSACKIE B SEROLOGY

GROUP	CASE NO.	TYPE VIRUS ISOLATED	DAYS AFTER ONSET SERUM COLLECTED		NEUTRALIZATION TITERS IN TISSUE CULTURE (HeLa) S_1/S_2				
			S_1*	S_2*	B_1	B_2	B_3	B_4	B_5
A1	1	B_2	3	21	< 16/ < 16	*< 16/64*	< 16/ < 16	< 16/ < 16	N.D.†
	2	B_2	5	23	< 16/ < 16	*< 16/64*	< 16/ < 16	< 16/ < 16	N.D.
	3	B_2	5	23	< 16/ < 16	*< 16/32*	< 16/ < 16	16/16	N.D.
	4	B_2	5	23	< 16/ < 16	*< 16/64*	< 16/ < 16	< 16/ < 16	N.D.
	5	B_2	5	12	< 16/ < 16	*< 16/32*	< 16/16	< 16/ < 16	N.D.
	6	B_3	4	> 30	< 16/ < 16	< 16/ < 16	*16/64*	< 16/ < 16	N.D.
	7	B_2	11	25	< 16/ < 16	< 16/ < 16	*16/64*	< 16/ < 16	N.D.
	8	B_1	19	90	*< 10/30-40*	< 10/ < 10	< 10/ < 10	< 10/ < 10	< 10/ < 10
	9	B_3	8	17	< 10/ < 10	< 10/ < 10	*20/80*	< 10/ < 10	< 10/ < 10
	10	B_1	1	8	30/ > 160	10/20	20/ < 10	< 10/ < 10	< 10/ < 10
	11	B_3	3	14	< 10/ < 10	< 10/ < 10	< 10/30	10/10	< 10/ < 10
	12	B_3	6	14	< 8/ < 16	< 8/ < 16	*32/256*	< 8/ < 16	< 8/ < 16
	13	B_4	1	10	< 8/ < 16	< 8/ < 16	< 8/ < 16	*128/1024*	< 8/ < 16
	14	B_4	3	> 30	< 8/ < 16	< 8/ < 16	< 8/ < 16	*< 8/512*	< 8/ < 16
	15	B_4	9	17	< 8/ < 16	< 8/ < 16	< 8/ < 16	*512/ > 1024*	< 8/ < 16
A2	16	B_2	3	12	*40/ > 80*	*< 10/40*	40/60	< 10/20	< 10/ < 10
	17	B_3	< 7	15	*< 10/ > 160*	< 10/ < 10	*< 10/160*	< 10/ < 10	< 10/ < 10
	18	B_3	2	11	*160/160*	10/ < 10	*10/60*	< 10/ < 10	< 10/ < 10
	19	B_3	7	25	*> 160/ > 160*	< 10/ < 10	*< 10/40*	< 10/ < 10	< 10/ < 10
	20	B_5	7	16	< 8/ < 16	< 8/ < 16	< 8/ < 16	*128/256*	*32/256*
B	21	B_2, B_4	3	6	< 16/ < 16	< 16/ < 16	< 16/ < 16	32/64	N.D.
	22	B_4	4	20	< 8/ < 16	< 8/ < 16	< 8/ < 16	*512/1024*	< 8/ < 16
C	23	B_3	9	17	< 16/ < 16	< 16/ < 16	*64/64*	16/16	N.D.
	24	B_2	0	15	< 16/ < 16	*32/32*	< 16/16	< 16/ < 16	N.D.
D	25	B_2	6	14	*10/ > 160*	*< 10/10*	*10/160*	20/20	< 10/ < 10

Positive serum titers in italics.
*S_1 = First serum sample; S_2 = second serum sample.
†N.D. = not done.

cells. No adaptation problems were encountered with subsequent passage in vitro. Furthermore, the tissue culture system employed seemed to provide a good menstruum for primary isolation and typing of viruses of the Coxsackie B group from clinical specimens. Viruses representative of all 5 immunologic types have been isolated from a variety of specimens, including 2 spinal fluids. A comparison of the frequency of isolations in HeLa and in the suckling mice showed that in some instances specimens yielded virus in only one of the systems employed. However, the agents so obtained could then be transmitted readily to the other host so that the failures cannot be ascribed to variant strains of virus. It is obvious that for optimal results both HeLa cells and suckling mice will have to be used. Virus strains, whether isolated in tissue culture or mice, could readily be identified by neutralization tests in tissue culture. The tissue culture system also proved to be satisfactory for determination of neutralizing antibody responses. In all of 25 cases studied, there was either a rise in titer against the homotypic virus isolated (23/25) or both acute and convalescent sera showed high titers to the specific agent isolated (2/25). Where a significant heterotypic response was noted as well (6/25) cases, antibodies against the B_1 and B_4 type were involved. This could possibly indicate a dual infection or an anamnestic response in the patient.

SUMMARY

Cultures of HeLa cells were employed for routine isolation and immunologic typing of viruses of the Coxsackie B group in human infections. Agents of all 5 types have been isolated from a variety of clinical specimens including spinal fluid. Some of the agents were more readily isolated in suckling mice than in HeLa cells and vice versa. The first passage strains thereafter were readily passed in either system.

Serologic evidence of infection was obtained in serum neutralization tests employing tissue culture-adapted viruses as antigen, and HeLa cell cultures as the host system.

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STUDIES ON ERYTHROPOIESIS

VI. Erythropoietin in Human Plasma

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THE mechanisms which regulate erythropoiesis have been under intensive investigation in recent years. Carnot and Deflandre[1] were the first to suggest a humoral mechanism was of importance in this regulation. Investigations prior to 1952, largely confined to animals, were summarized in the extensive review by Grant and Root,[2] and at that time evidence favored the humoral control of erythropoiesis. The factor responsible for this control has been referred to as erythropoietin by Bonsdorff and Jalavisto.[3]

A significant advance was made by Borsook, Graybiel, Keighley, and Windsor[4] when they demonstrated an erythropoietic stimulating factor in the filtrate obtained from heat denatured anemic rabbit plasma. Although obtained from anemic rabbits, this filtrate could be assayed in the normal rat. The appearance of an erythropoietic factor in the plasma of rats or rabbits made anemic by phlebotomy or injection of phenylhydrazine has been demonstrated by numerous investigators.[5-9]

Recent investigators have turned their attention to the significance of erythropoietin in the human being. Contopoulos and his co-workers[10] have demonstrated a plasma factor from blood of patients with polycythemia vera which is capable of stimulating erythropoiesis in the rat. Linman and Bethell[11] have demonstrated stimulation of erythropoiesis in the normal rat by injections of extracts of plasma from patients with polycythemia vera and secondary polycythemia. Pilero and co-workers[12] have found an erythrocyte stimulating factor in the plasma of patients with Cooley's anemia and sickle-cell anemia. Their report also summarizes previous work with human plasma by other investigators.

The purpose of this report is to record the demonstration of erythropoietin in the extract of heat denatured plasma obtained from anemic patients. This demonstration has been facilitated by the development of an assay procedure by Plzak, Fried, Jacobson, and Bethard[13] dependent on the increased incorporation of radioiron into erythrocytes of rats stimulated by the intravenous injection of erythropoietin. Although Gordon, Piliero, Tannenbaum, and Siegel[14] had previously demonstrated that the hypophysectomized rat was capable of responding to anemic rabbit plasma extract with increases of peripheral red cell, hemoglobin, hematocrit, and reticulocyte values, Jacobson and his co-workers[15, 16] first clearly demonstrated that the hypophysectomized

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rat was an extremely sensitive animal for assay of erythropoietin activity and that a dramatic increase of Fe^{59} incorporation into the animal's erythrocytes followed only 2 or 3 injections of anemic rat plasma. The significance of this assay method rests in the great increase of red cell Fe^{59} incorporation, often several hundred per cent greater than that produced by normal plasma, requiring only small amounts of anemic plasma. It has, therefore, become possible to avoid lengthy assays in which small rises in peripheral hemoglobin, red count, or hematocrit following many injections of a plasma extract represent activity of the extract being tested. In the present study this assay procedure was used in an attempt to determine the presence of erythropoietin in normal and anemic human plasma.

METHODS

Fifty to 150 ml. of blood from anemic patients was collected in 250 ml. bottles each containing 1 ml. (1,000 units) of heparin. Normal control blood was collected in 500 ml. bottles each containing 2 ml. of heparin. Immediately after collection the plasma was separated by centrifugation in the cold, removed, and stored in the Deepfreeze until extraction.

Plasma was extracted by the boiling method as described by Borsook and co-workers.[4] The plasma was brought to a pH of 5.5 by addition of 0.1 normal hydrochloric acid, put into a boiling water bath for 10 minutes, and filtered. The precipitate was washed twice with an equal volume of distilled water, all filtrates being pooled. Employing a rotary evaporator, the filtrate was then concentrated to the volume of the original plasma specimen. Since normal plasma failed to demonstrate erythropoietic stimulating activity, we considered the possibility that erythropoietin might be present in titers insufficient to demonstrate in normal plasma by the assay system employed, and some specimens of normal plasma were, therefore, concentrated to one-tenth the original volume. Such specimens were then dialyzed for 48 hours in order to remove the excessive amounts of electrolytes present as a result of concentration. After concentration to the desired volume, the pH was adjusted to 7.5 by the addition of 0.1 normal sodium hydroxide.

An extract of normal plasma was also prepared using perchloric acid. A volume of 10 per cent perchloric acid equal to the plasma specimen was added to the plasma slowly with continuous stirring. The precipitate was washed two times with 5 per cent perchloric acid; each washing was with a volume equal to one-half the original plasma volume. The supernatant fluid and washings were placed in a Visking Casing bag, dialyzed against distilled water for 72 hours, concentrated to the desired volume, and the pH was adjusted to 7.5 by the addition of 0.1 N sodium hydroxide.

Hypophysectomized male Sprague-Dawley rats, 130 to 180 grams in weight, were used. The animals in each assay were 8 to 15 days posthypophysectomy when the assay was begun. On 2 successive days, 2 ml. of anemic plasma extract were injected in the tail vein while the animals were under light ether anesthesia. On the third day a tracer dose of 1 to 2 μc of Fe^{59} (activity approximately 2,000 mc. per gram of iron) in saline, buffered to pH 7.5 with sodium citrate, was injected. Sixteen hours after iron injection, 1 ml. of blood was obtained by cardiac puncture for assay. Between successive cardiac punctures, the syringe used was rinsed in normal saline and finally in heparin (1,000 units per milliliter). The thin film of heparin left in the syringe was sufficient to prevent clotting.

Blood samples and two aliquots of the standard prepared at the time of radioiron injection were counted in a Nancy-Wood scintillation counter. Assuming a blood volume of 5 per cent of body weight,[13] and correcting counts for background, the per cent Fe^{59} incorporation in red cells at 16 hours was calculated. Per cent iron incorporation =

$$\frac{\text{net counts of blood per minute/ml.} \times \text{body weight in grams} \times 0.05}{\text{net counts of standard/ml.}}$$

Results are expressed as average per cent uptake ± one standard deviation. Starved, normal, male Sprague-Dawley rats of the same age and weight as the hypophysectomized

animals were also occasionally used for assay.[17] These animals had no food for 24 hours prior to the beginning of the assay, and were maintained in the starved condition throughout the assay, water being given ad libitum.

At least one normal plasma control and a saline control assay was run every 2 weeks over the duration of the entire study. A normal plasma control was not, however, run with every assay.

RESULTS

Studies on Normal Plasma.—The average uptake obtained when the extracts of individual heat denatured plasma specimens from 10 normal donors with hemoglobin above 14 Gm. per cent was used in hypophysectomized rats was 3.1 per cent; this value does not differ appreciably from an uptake of 3.4 per cent when normal saline was used. In starved rats, 3.5 per cent uptake was obtained with extract of normal plasma from 6 donors, and an uptake of 3.2 per cent followed the injection of normal saline. These results are summarized in Table I. It seemed likely to us that normal plasma was not devoid

TABLE I. INFLUENCE OF EXTRACT OF NORMAL HUMAN PLASMA ON PER CENT OF Fe^{59} INCORPORATION IN RAT ERYTHROCYTES*

Assay of heat denatured plasma from 10 normal human donors assayed in 49 hypophysectomized rats	3.1 ± 1.1%
Saline control in 40 hypophysectomized rats	3.4 ± 1.4%
Assay of heat denatured plasma from 5 normal human donors assayed in 22 hypophysectomized rats	3.5 ± 1.7%
Saline control in 25 starved rats	3.2 ± 1.1%

*Average values ± one standard deviation.

of erythropoietin, but that the concentration was so small as to escape detection by this assay. To test this possibility, after precipitation of proteins by heat or perchloric acid, the supernatant fluid was concentrated 10 times. The incorporation of Fe^{59} into red cells was sufficiently increased, when compared to incorporation following injection of saline or unconcentrated plasma extracts to indicate that erythropoietin is present in normal plasma. These results are summarized in Table II.

TABLE II. ERYTHROPOIETIC ACTIVITY OF FRACTIONATED CONCENTRATED NORMAL PLASMA (5 Hypophysectomized Rats in Each Group)

Heat denatured	10 × normal concentration, dialyzed	12.6 ± 2.1%
	Normal concentration, dialyzed	2.6 ± 1.2%
Perchloric acid	10 × normal concentration, dialyzed	7.6 ± 2.5%
	Normal concentration, dialyzed	2.8 ± 1.3%

Assay of Plasma From Anemic Patients.—Twenty-one plasma specimens were obtained from 18 patients with anemia. Twelve were positive, and 9 were considered equivocal or negative. At this time, we prefer to consider only those assays averaging 7 per cent or more in hypophysectomized rats as positive (control = 3.1 per cent), although undoubtedly lower values could be considered positive if larger numbers of animals were used in each assay. Results of these assays are summarized in Table III.

In the first patient, hypoplastic anemia was secondary to Atabrine which had been administered on 3 occasions for treatment of chronic discoid lupus

TABLE III. PER CENT Fe^{59} INCORPORATION IN RAT ERYTHROCYTES AFTER INJECTION OF BOILED EXTRACT OF ANEMIC PLASMA

NO.	AGE	DIAGNOSIS	HEMOGLOBIN	HEMATOCRIT	RETICULOCYTE COUNT (%)	R.B.C.	ASSAY		RESULT
							HYPOPHYSECTOMIZED RAT	STARVED RAT	
1	40	Hypoplastic anemia, specimen 1	5.2	14.0	2.1	1,370,000	18.3 ± 5.7 (7)		+
		Hypoplastic anemia, specimen 2	6.0	19.0	3.8	2,000,000	8.0 ± 2.5 (5)	10.8 ± 2.0 (5)	+
		Hypoplastic anemia, specimen 3	10.8			2,820,000	12.9 ± 1.1 (4)	12.3 ± 2.7 (5)	+
		Hypoplastic anemia, specimen 4	9.3		8.9		4.6 ± 1.4 (5)		−
							4.4 ± 0.9 (5)		−
2	40	Hypoplastic anemia	6.9	13.0			7.2 ± 1.5 (5)		+
3	43	Hypoplastic anemia	3.2	9.0	0.6	1,210,000	26.9 ± 3.6 (5)		+
4	54	Pernicious anemia	7.3		2.0	2,100,000	7.2 ± 2.2 (5)		+
5	38	Hemolytic anemia	9.6	30.0	8.8		8.2 ± 2.1 (6)		+
6	50	Chronic granulocytic leukemia	5.1	16.5	0	2,150,000	17.4 ± 2.7 (5)		+
7	47	Chronic granulocytic leukemia	6.1		0.8	1,830,000	22.7 (2)	7.6 ± 2.9 (6)	+
8	39	Chronic granulocytic leukemia	6.0	21.0	0	2,950,000	9.2 ± 1.0 (5)		+
9	49	Subacute granulocytic leukemia	7.3	20.0	1.2	2,100,000	10.2 ± 5.2 (4)		+
10	54	Subacute granulocytic leukemia	8.5	29.0	2.8	2,700,000	6.3 ± 2.4 (5)		−
11	26	Chronic granulocytic leukemia	10.6	36.0	4.5	3,800,000	1.8 ± 0.6 (6)	3.1 ± 0.3 (6)	−
12	46	Acute gastrointestinal hemorrhage	8.3	24.0	2.7	2,760,000	7.5 ± 2.2 (6)		+
13	59	Hodgkin's disease with hemolytic anemia	4.1	13.0	0.8	1,510,000	7.6 ± 1.0 (6)		+
14	66	Carcinoma of stomach	8.8	25.5			3.2 ± 0.4 (5)		−
15	62	Islet cell carcinoma of pancreas	7.7	22.0	1.1	2,560,000	4.5 ± 1.4 (5)		−
16	16	Chronic glomerulonephritis	5.5	15.5			2.8 ± 0.9 (6)		−
17	41	Sickle cell anemia	7.7			2,110,000	4.3 ± 1.1 (5)		−
18	64	Plasmocytic myeloma	7.3		5.5		4.8 ± 0.8 (5)		−
							4.3 ± 1.7 (5)		−

Number in parentheses refers to number of animals in each assay.
+ = Positive assay.
− = Negative assay.

erythematosus of the face and scalp, each course being terminated because of the development of anemia. The patient was referred to the University of Chicago Clinics when, after discontinuance of the last course of Atabrine, the red count and hemoglobin continued to fall. There was no evidence for kidney disease or other systemic disease in the history, physical examination, or laboratory studies. Plasma was obtained on 4 occasions during the course of the illness. The first 2 specimens were strongly positive. The plasma for each of these assays was drawn immediately prior to transfusion of whole blood. The third plasma sample was obtained 4 days after transfusion of blood to hemoglobin of 10.8 Gm. per cent, and on this occasion, activity was no longer demonstrable. The patient subsequently improved and the hemoglobin fell slowly and has temporarily stabilized at a level above 9 Gm. The last plasma specimen was obtained at this time, and again was negative.

The second patient was critically ill with a hypoplastic anemia of unknown etiology when admitted to the hospital, and for this reason, phlebotomy was considered contraindicated. However, from blood removed from the heart at post-mortem examination, 6 hours after death, it was possible to obtain plasma which still yielded a positive result, the average uptake in hypophysectomized rats being 7.2 per cent.

The third patient with hypoplastic anemia of unknown etiology, of 6 months' duration, on 2 occasions revealed virtually complete aplasia on both smear and section of the material obtained. Twenty-six and nine-tenths per cent of the tracer dose of $Fe^{59}Cl_3$ was incorporated in the rat red cells. It has been shown that radioactive iron is removed from the plasma within 4 to 6 hours after its injection,[18] but this amount of Fe^{59} incorporation was so spectacular that we wished to be sure we were measuring Fe^{59} intimately associated with erythrocytes, and not plasma iron mobilized from stores into plasma. Therefore, to each 1 ml. blood specimen, 5 ml. of normal saline was added, and after centrifugation, 5 ml. of supernatant fluid (saline and plasma) was removed. The remaining 1 ml., consisting of red cells, some plasma, and saline, was again counted and the calculated uptake was 26.4 per cent. The close agreement of activity of samples before and after washing demonstrates practically all the Fe^{59} is associated with the red cells, and little is free in the plasma.

Plasma from a primaquine-sensitive subject* was obtained for assay on the fifth day of primaquine ingestion. Following administration of the drug, the subject responded with an acute hemolytic episode with the hematocrit falling from 45 to 30 per cent in 5 days. The plasma of this patient was positive for erythropoietin activity, averaging 8.2 per cent.

Six leukemic patients are included in this study to date. The extract of plasma from Patient 6 assays strongly positive. Although 2 of 5 animals showed a strong positive assay to extract of plasma from Patient 7, the other 3 animals in this assay group died. Sufficient plasma was available for assay in starved rats, and although the magnitude of the increase of Fe^{59} incorporation into red cells is smaller, the result is positive. Plasmas from Patients 8

*Kindly supplied by Drs. Alf Alving and Stanley Schrier.

and 9 were positive. In keeping with our arbitrary assumption that incorporation of 7 per cent or more of the administered dose is required before an assay be considered positive, plasma from Patient 10 is classified as negative, although 6.3 per cent incorporation clearly exceeds any of the control values. Patient 11 had chronic granulocytic leukemia accompanied by mild anemia. Assays of extracts of his plasma were negative in both hypophysectomized and starved rats.

Positive results were obtained when extracts of plasma from patients with pernicious anemia, acute gastrointestinal hemorrhage, and Hodgkin's disease with anemia were assayed.

Diagnoses of patients whose plasma extract was negative or equivocal are enumerated in Table III. Only Patients 11 and 16 were in a good state of nutrition. Patient 17 was in a poor state of nutrition and has persistently in the last year had a hemoglobin under 9 Gm. The remaining patients were cachectic and considered to be approaching terminal status at the time blood was obtained for assay.

DISCUSSION

The rats used for assay in this study have plasma volumes of approximately 3.5 ml. Therefore, it is logical to inquire as to why the erythropoietin in 4 ml. of normal plasma is not sufficient to stimulate erythropoiesis. Although we cannot answer this question at the present time, we believe there is loss of activity of plasma after removal from the body.[19] We are not now able to ascertain exactly how much erythropoietin is lost in the extraction employed here, but preliminary studies in our laboratories suggest about 50 per cent loss.[19] If the rate of in vivo turnover is great, it may be that several times that amount of erythropoietin present in the normal rat at any one time would have to be given to the hypophysectomized or starved rat over a 2-day period in order to increase erythropoiesis. And finally, if under physiologic circumstances the rat should have a higher concentration of erythropoietin than the human, concentrations which would be normal for the latter would not be sufficient to produce an optimal response in the rat.

It is apparent that without concentrating the plasma extract, an active erythropoietic stimulating principle can be demonstrated in the heat denatured extract of plasma from human beings with a variety of anemias. It is not entirely clear at this time why a high titer of erythropoietin cannot be demonstrated in the plasma of all anemic subjects. A recent abstract by Prentiss and Mirand[20] reports demonstration of erythropoietic activity in plasma of 10 to 15 patients with anemia, using the assay procedure first employed by Fried, Plzak, Jacobson, and Goldwasser.[16] The diagnoses associated with the anemias were not stated. In their study the extract was concentrated 3 times. It is probable that some of our extracts which were negative would have been positive had the plasma been concentrated. We chose not to concentrate plasma extracts in this initial clinical study for two reasons. First, we have been reluctant to remove routinely more than 50 ml. of blood from severely anemic patients until we had thoroughly evaluated the results we could obtain by assaying a series of anemic plasmas without concentrating them. Second, if it is to give the most meaningful results, concentration of anemic

plasma should be as many times as is required to demonstrate activity in normal, nonanemic plasma. Although we have demonstrated activity in normal plasma extracted by perchloric acid precipitation or heating and concentrated 10 times, we have not yet determined in a large series of normal plasma specimens the minimum number of times the filtrate from heat denatured normal plasma must be concentrated in order to demonstrate activity. Concentration of extracts of anemic plasma an arbitrary number of times may yield a few more positive assays. It will not, however, make negative results any more meaningful. Negative assays will be significant only when they indicate decreased concentrations of erythropoietin in plasma of anemic subjects. We have chosen not to concentrate extracts of anemic plasma until we have determined the number of times we must concentrate them in order to make a negative result indicative of decreased erythropoietin concentrations.

It may be, however, that failure to demonstrate erythropoietin in the plasma of all anemic patients cannot in every instance be attributable to insensitivity of the assay method. It appears possible to us that some anemic states may be accompanied by and to some degree attributable to a decreased concentration of erythropoietin, with a new steady state being established at hemoglobin levels less than normal.

Bethard and Wissler[21] have demonstrated reduction of the rate of erythropoiesis in protein depleted rats as measured by Fe^{59} uptake, and Fried, Plzak, Jacobson, and Goldwasser[17] have reported a decreased rate of erythropoiesis in acutely starved animals. It may be that in some patients, as in Patients 14 and 15 with carcinoma, starvation contributes to decreased erythropoiesis by decreasing erythropoietin production. Finally, in light of recent evidence reported by Jacobson and associates[22] suggesting that erythropoietin is produced in the kidney, it is interesting to speculate that decreased production of erythropoietin may have contributed to failure to demonstrate activity in the extract of anemic plasma of a patient with chronic renal disease. Excessive excretion of erythropoietin in chronic renal disease is still another possibility which warrants investigation, particularly since an erythropoietic stimulating factor has been demonstrated in the urine of an anemic patient by Piliero and co-workers.[12]

The appearance of increased concentration of erythropoietin in animals following bleeding or hemolysis,[4-9, 13-16] the high concentrations of erythropoietin in several of our patients, and the prompt disappearance of the high concentration in one patient following blood transfusion suggests to us the possibility that the homeostatic mechanism by which a patient responds to anemia by increased erythropoiesis is mediated through production of increased amounts of erythropoietin. If future investigation substantiates this hypothesis, it is apparent the assay method as now employed is not very sensitive, since reticulocyte counts as high as 8.9 per cent were found in patients whose plasma failed to demonstrate activity on bio-assay.

It is clear that a relationship does not of necessity exist between severity of anemia and erythropoietin concentration or degree of reticulocytosis and

erythropoietin concentration. A hemoglobin of 5.2 is associated with a plasma erythropoietin concentration sufficient to increase Fe^{59} incorporation sixfold in the case of hypoplastic anemia, while the plasma of a nephritic patient with a comparable anemia failed to demonstrate activity by the method employed. The bone marrow may be refractory to the stimulus produced by an increased erythropoietin content, as in Patient 5 with leukemia where no reticulocytes were present, while levels of erythropoietin sufficiently below the threshold of detection by this method may still be associated with a reticulocytosis, as in Patients 1, 11, and 18.

We are led to postulate that patients with severe anemia have a high plasma titer of erythropoietin unless its production is decreased or its destruction or excretion is increased. In those instances where the patient will spontaneously correct the low hemoglobin levels, as following acute hemorrhage and drug induced hemolytic anemia, we believe the elevated erythropoietin concentration will be found to decrease as the hemoglobin rises. The erythropoietin content may be expected to remain high in anemias which are not correctable by the unaided homeostatic body mechanisms, but will probably be found to decrease following blood transfusion to a normal level or following rise of hemoglobin to a normal level, as in pernicious anemia adequately treated with B_{12}. Finally, we believe future work may indicate some anemias will be attributable to decreased or absent erythropoietin production. The recent investigation of Pilero and co-workers[12] suggests primary red cell anemia of children may be such a disease. Since cobalt appears to act by increasing the production of erythropoietin,[23] it is reasonable to anticipate anemias associated with renal disease, chronic infection, neoplasm, and rheumatoid arthritis, all responsive to cobalt administration[24-28] may be attributed to low plasma erythropoietin concentrations and may, therefore, respond to erythropoietin when a preparation sufficiently potent and pure enough to permit clinical trials becomes available. The decreased rate of iron incorporated in the red cells of starved rats and the response in these animals to anemic rat plasma[17] suggest anemias accompanying starvation and cachectic states may be at least partially the result of low plasma erythropoietic concentrations.

SUMMARY

1. Using the incorporation of Fe^{59} into erythrocytes of hypophysectomized or starved male Sprague-Dawley rats, erythropoietin has been demonstrated in normal plasma. This required tenfold concentration when plasma extracts are prepared by heat denaturization or perchloric acid precipitation.

2. Without concentrating the plasma extracts, erythropoietin has been demonstrated in heat denatured plasma extracts of 10 anemic subjects.

3. Hypoplastic anemia, in 3 patients, cannot be attributed to decreased erythropoietin concentration.

4. High erythropoietin concentrations in the blood of one patient with hypoplastic anemia fell promptly following blood transfusion.

5. Failure to demonstrate activity by this method is not necessarily indicative of decreased erythropoietin concentrations.

6. The results of this study are consistent with the hypothesis, first formulated by Carnot and Deflandre in 1906, that the rate of erythropoiesis is mediated by a humoral mechanism.

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OBSERVATIONS ON THE NATURE OF THE ERYTHROPOIETIC SERUM FACTOR

II. Erythropoietic Activity of Serum and Bone Marrow After Time Limited Exposure to Anemic and Hypoxic Anoxia

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THE rate of red cell production, although conditioned by many known minerals, vitamins, and hormones, appears to be controlled by the tissue tension of oxygen. The physiologic mechanism responsible for this control is unknown. However, it has been suggested that a humoral erythropoietic factor might serve as a mediator between a specific oxygen sensitive tissue and the bone marrow.[1]

Recently, this concept has received experimental support by the demonstration of an erythropoietic factor in plasma and serum from animals exposed to anemic and hypoxic anoxia (see review by Erslev[2]). The erythropoietic factor has not been isolated or purified as yet, but it has been found to be present in a nondialyzable fraction of the protein-moiety of serum.[3]

In order to define further the physiologic mechanism of the control of erythropoiesis, the time relationship between the induction of tissue anoxia and the appearance and persistence of erythropoietic factor in serum was studied.

METHODS

Adult, white male New Zealand rabbits weighing 2.2 to 3.2 kilograms were used throughout. Anemic anoxia was induced by a single bleeding of 20 ml. per kilogram body weight. In order to minimize circulatory adjustment, the bleeding was promptly followed by an intravenous infusion of an equal volume of 6 per cent dextran in saline. The anemia was terminated by reinfusion of the blood removed for which heparin was used as anticoagulant.

Hypoxic anoxia was induced by exposing the animals to 10 or 11 per cent oxygen at normal barometric pressure in an airtight chamber.[4] The oxygen level was kept constant by admitting compressed room air and pure nitrogen at flow rates sufficient to provide a total air flow per minute of 4 liters per animal. An electric fan maintained even oxygen concentration in the chamber, while calcium chloride and calcium hydroxide removed excessive moisture and carbon dioxide.

The serum obtained within a few minutes after the termination of the anoxic periods was bio-assayed for erythropoietic factor. For each bio-assay 3 to 4 animals were exsanguinated and their serum pooled. The technique used in the bio-assay has been described in detail in a previous publication.[3] In short, 50 ml. of the serum to be tested was

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infused intravenously into normal rabbits once a day for 4 days. Reticulocyte counts and hemoglobin concentrations were followed for 7 days, and the magnitude of the reticulocyte response was taken as an index of the erythropoietic activity of the infused serum.

Reticulocyte counts were made from blood smears on cover glasses, prepared with brilliant cresyl blue. The smears were counterstained with Wright's Stain. One thousand red cells were counted. During anemic periods the reticulocyte percentages were corrected for the fall in the total number of red cells, and expressed in per cent of the initial red cell count.

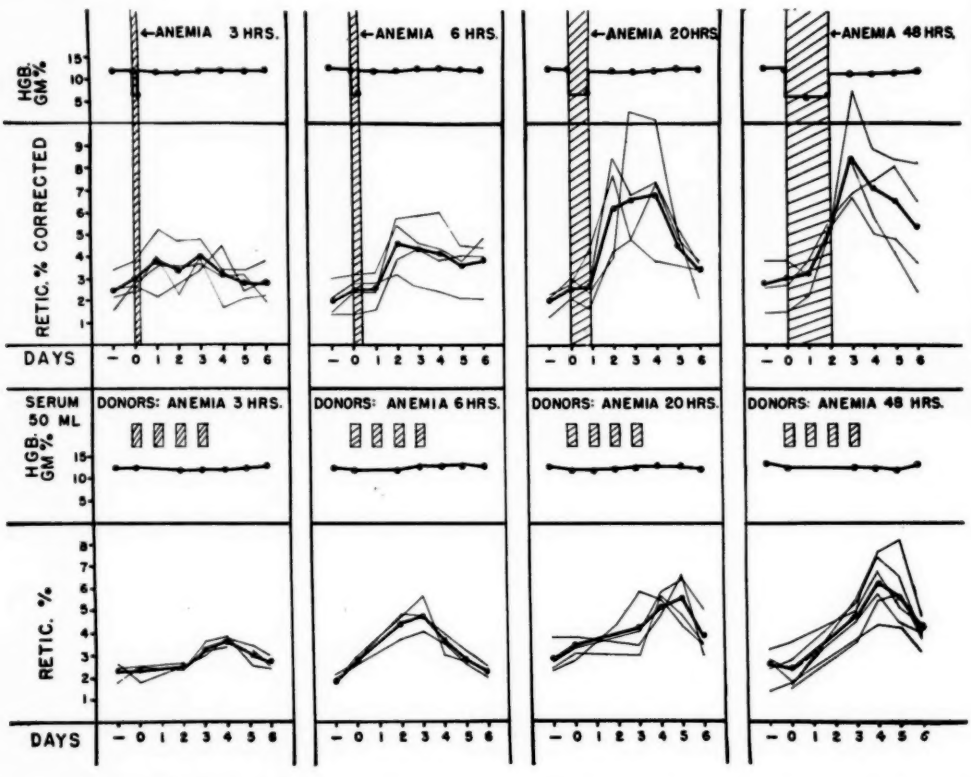

Fig. 1.—Charts in upper row: Mean hemoglobin concentrations and mean and individual reticulocyte percentages of normal rabbits bled 20 ml. per kilogram with immediate restoration of blood volume by 6 per cent dextran in saline. In different groups of rabbits the anemia was terminated 3, 6, 20, or 48 hours later by reinfusion of the previously removed blood. During the anemic periods reticulocyte percentages were corrected for the decrease in the number of red blood cells. Charts in lower row: Mean hemoglobin concentrations and means and individual reticulocyte counts of normal rabbits receiving four daily infusions of 50 ml. of serum. Serum was obtained from groups of rabbits after 3, 6, 20, or 48 hours of anemic anoxia.

RESULTS

1. *Reticulocyte Response to Anemic Anoxia.*—Groups of normal rabbits were rendered anemic by blood loss (Hgb. $<$ 7.0 Gm. per cent) for 3, 6, 20, or 48 hours. After such intervals the anemia was terminated by reinfusion of the blood drawn. The individual and mean reticulocyte responses to this acute, time-limited anemia are charted in the upper portion of Fig. 1, along with the mean hemoglobin concentrations. A moderate increase in the number of reticulocytes was observed in most animals kept anemic for 6 hours. Anemia maintained for 20 hours induced a significant reticulocytosis, although, less pronounced than the response observed after 48 hours of anemia.

2. *Erythropoietic Activity of "Anemic" Serum.*—Groups of rabbits rendered anemic by blood loss (Hgb. < 7.0 Gm. per cent) for 3, 6, 20, or 48 hours were bled to death and the serum from each group bio-assayed for erythropoietic activity. The results are shown in the lower portion of Fig. 1. Serum from animals kept anemic for 3 hours was only questionably active, while serum from animals kept anemic for 6 hours showed a definite erythropoietic activity. Even greater activity was present in serum from animals kept anemic for 20 hours and 48 hours. Serum from animals with a hemoglobin concentration maintained at less than 7 Gm. per cent for 5 days, revealed erythropoietic activity similar to the activity found after 20 and 48 hours of anemia (Fig. 2, *A*).

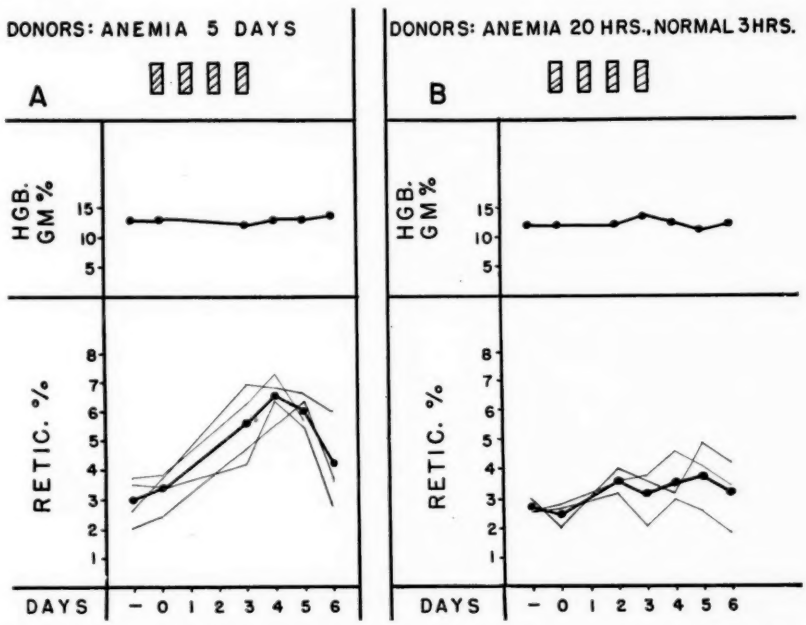

Fig. 2.—Mean hemoglobin concentrations and mean and individual reticulocyte counts of normal rabbits receiving four daily infusions of 50 ml. of serum. *A*, Serum obtained from rabbits after five days of sustained anemia (Hemoglobin < 7.0 Gm. per cent). *B*, Serum obtained from rabbits 3 hours after the termination of a 20-hour period of anemic anoxia.

3. *Erythropoietic Activity of Serum After Termination of Anemic Anoxia.*—Rabbits rendered anemic by blood loss (Hgb < 7.0 Gm. per cent) for 20 hours, were bled to death 3 hours after the anemic anoxia had been terminated by means of reinfusion of the previously removed blood. Bio-assay of this serum revealed an almost complete disappearance of erythropoietic activity (Fig. 2, *B*).

4. *Reticulocyte Response to Hypoxic Anoxia.*—Groups of normal rabbits were exposed to 11 per cent oxygen for 3, 6, or 20 hours and their erythropoietic response was compared with results obtained in a previously reported study, with rabbits exposed to 10 per cent oxygen for 48 hours.[4] As shown in the upper portion of Fig. 3 no reticulocyte response followed 3 and 6 hours of hypoxia. Hypoxia lasting 20 hours and 48 hours were followed by a definite

increase in the number of reticulocytes. However, comparison with Fig. 1 shows that the response to hypoxic anemia in each instance was less than the response to anemic anoxia of corresponding duration.

5. *Erythropoietic Activity of "Hypoxic" Serum.*—Groups of rabbits exposed to 11 per cent oxygen for 3, 6, or 20 hours and another group of rabbits exposed to 10 per cent oxygen for 48 hours[4] were bled to death and the sera from each group of animals were bio-assayed for erythropoietic activity. As shown in the lower portion of Fig. 3 the activity of these sera corresponded closely to that of sera from rabbits kept anemic for corresponding periods of time.

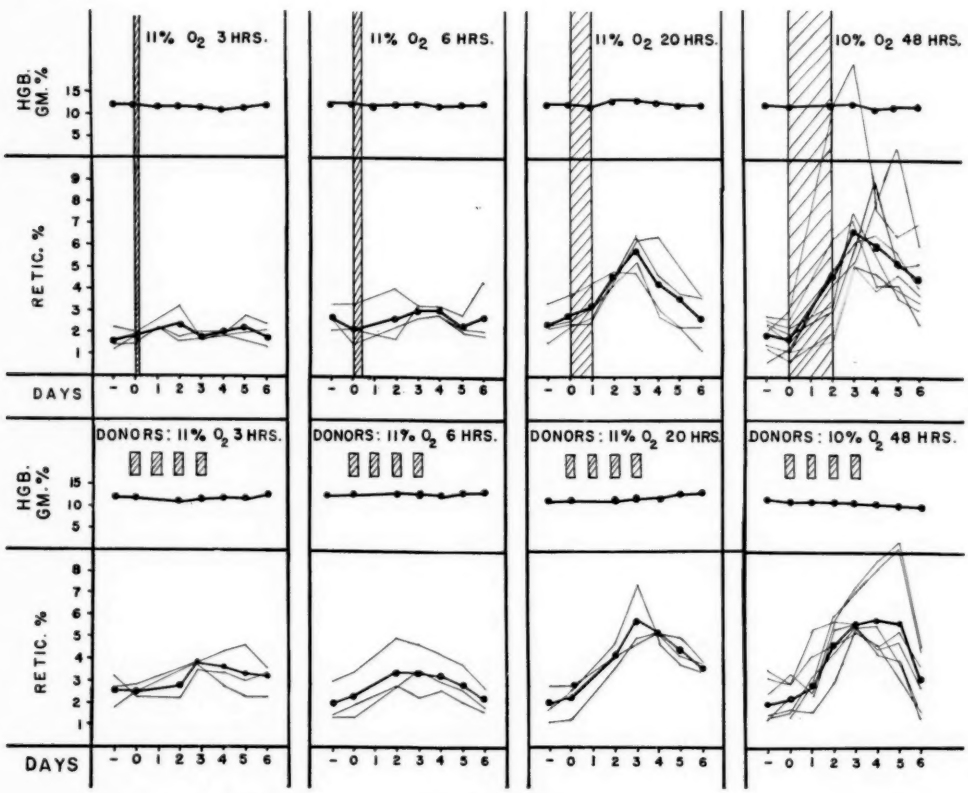

Fig. 3.—Charts in upper row: Mean hemoglobin concentration and mean and individual reticulocyte counts of normal rabbits exposed to ambient air with an oxygen concentration of 10 or 11 per cent. In different groups of rabbits the hypoxia was terminated after 3, 6, 20, or 48 hours. Charts in lower row: Mean hemoglobin concentration and mean and individual reticulocyte counts of normal rabbits receiving four daily infusions of 50 ml. of serum. Serum was obtained from groups of rabbits after 3, 6, 20, or 48 hours of hypoxic anoxia.

DISCUSSION

A reduction in the oxygen carrying capacity of blood, or a reduction in the oxygen saturation of its hemoglobin will result in a decrease in the tissue tension of oxygen.[5] Under physiologic conditions, this decrease is associated with an increased rate of red cell production and with the appearance of erythropoietic factor in the serum.

In the study reported here, an acute anemic anoxia with a reduction in hemoglobin concentration to approximately 50 per cent of normal was induced by means of a single bleeding and maintained for 3, 6, 20, or 48 hours. Changes in the number of reticulocytes during the next 6 days were used to evaluate the effect of these periods of anoxia on the bone marrow. A slight increase in erythropoietic activity was noticeable after 3 hours of anemic anoxia, with a further progressive increase as the periods of anoxia were prolonged. The reticulocyte responses occurred after a delay of 48 hours without evidence of a significant immediate release of reticulocytes.

Erythropoietic activity of serum obtained at the end of the anoxic periods was found to be slight, but measurable after 3 hours, was definite after 6 hours and very significant after 20 hours of anemic anoxia. Anemic anoxia maintained for 48 hours or for 5 days, was associated with the same degree of erythropoietic activity of serum, as after 20 hours. After termination of the anemic periods, the erythropoietic activity of serum was found to have almost completely disappeared in three hours.

From these data, it is impossible to state definitely whether the appearance of erythropoietic activity in serum precedes increased bone marrow activity, or whether the erythropoietic factor merely reflects increased mitotic activity of the normoblastic tissue in the bone marrow. However, it has been shown that anemic anoxia in animals that have received nitrogen mustard is associated with erythropoietic activity of the serum, despite complete absence of erythropoietic activity of the bone marrow.[3] Consequently, it seems likely that erythropoietic activity of serum precedes erythropoietic activity of the bone marrow. This would support the hypothesis that tissue anoxia controls the production or release of an erythropoietic factor, which in turn controls the rate of red cell production. Further support for this hypothesis comes from the observation that erythropoietic activity of serum, induced by anemic anoxia, disappears rapidly after correction of the anemia.

Acute hypoxic anoxia was induced by exposing animals to air mixtures containing 10 or 11 per cent of oxygen. Hypoxic anoxia lasting 3 and 6 hours was not followed by any significant increase in the number of reticulocytes. Hypoxic anoxia of 20 hours' duration was followed by a definite reticulocyte response, although less than the response observed[4] after 48 hours of hypoxic anoxia. The increase in erythropoietic activity of the bone marrow was less in all groups than the increase observed after anemic anoxia of a corresponding duration. This difference in response might be due to a difference in the degree of tissue anoxia. It is very possible that a 50 per cent reduction in hemoglobin concentration will result in a more severe tissue anoxia than a 50 per cent reduction in the oxygen concentration of ambient air. However, the anoxic insult in both the anemic animals and in the hypoxic animals was close to the limit of tolerance, with a 10 to 15 per cent mortality. Furthermore, the erythropoietic activity of sera obtained immediately after corresponding periods of hypoxic anoxia and anemic anoxia were strikingly similar. These considerations suggest that the degree of anoxia in the anemic animals

and in the hypoxic animals were approximately the same. The difference in bone marrow response could conceivably be related to the fact that compensatory hyperventilation and alkalosis occur in animals with hypoxic anoxia, and not in animals with anemic anoxia.

The tissue or organ in which a low tissue tension of oxygen causes an elaboration of the erythropoietic factor is still unknown. Saline extracts from various tissues including bone marrow, spleen, liver, thymus, lung, and kidneys obtained from anemic animals have not been shown to be erythropoietically active.[6]

Pituitary extracts have been shown to possess some erythropoietic activity.[7, 8] However, it appears unlikely that the erythropoietic factor is produced in the pituitary gland, since this factor has been found in the serum of hypophysectomized animals exposed to anemic anoxia.[9, 10] Recently, Jacobson and co-workers[11] have suggested that the erythropoietic factor is produced in the kidneys. Unfortunately, observations on uremic rabbits do not support this attractive idea.[12] First, serum from rabbits rendered both anemic and uremic showed a low level of erythropoietic factor whether the uremia was induced by bilateral nephrectomy or by bilateral ureter ligation. Furthermore, the bone marrow of nephrectomized rabbits continued to be active despite the complete absence of kidney tissue. That the factor is not elaborated in the blood stream itself is suggested by the fact that plasma from normal whole blood, equilibrated in vitro against pure nitrogen for 24 hours, failed to show erythropoietic activity. Likewise, plasma from whole blood of anemic animals remained erythropoietically active despite equilibration against air mixtures with 21 per cent oxygen and 100 per cent oxygen for 24 hours.[6]

That some specific tissue system must be responsible for the elaboration of the erythropoietic factor seems obvious, but much more work and probably a less laborious bio-assay technique will be necessary before this system and the physiologic mechanism responsible for the production of erythropoietic factor can be identified.

SUMMARY

Anemic anoxia with a 50 per cent reduction in hemoglobin concentration or hypoxic anoxia with a 50 per cent reduction in the oxygen tension of ambient air were induced and maintained in groups of normal rabbits for 3, 6, 20, or 48 hours, respectively. The reticulocyte response to the anoxia was determined in each animal, as well as the erythropoietic activity of serum obtained at the end of the anoxic periods.

In animals with anemic anoxia a moderate increase in the number of reticulocytes was found after 6 hours of anemia, with progressively greater increases in the reticulocyte response after 20 and 48 hours of anemia. In animals with hypoxic anoxia there was no response after 3 and 6 hours of hypoxia, while 20 and 48 hours induced significant reticulocyte responses. However, these were lower than in the animals kept anemic for corresponding durations of time.

The erythropoietic activity of the pooled serum obtained at the end of the anemic periods was found to be questionable after 3 hours, definite after 6 hours, and maximal after 20 hours. Maintenance of the anemic state for 48 hours or for 5 days did not change the activity of the serum. The erythropoietic activity of serum from animals with hypoxic anoxia corresponded closely to the activity of serum from rabbits kept anemic for the same length of time.

Serum obtained 3 hours after the termination of a 20-hour period of anemic anoxia was found to have only minimal erythropoietic activity.

I wish to thank Miss Elva Ruiz for valuable assistance in this study.

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NEONATAL SEROLOGIC DIAGNOSIS OF HEMOLYTIC DISEASE OF THE NEWBORN CAUSED BY ABO INCOMPATIBILITY

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THE diagnosis of hemolytic disease of the newborn (HDN) is considerably more difficult if the disease is caused presumably by maternal isosensitization to the A or B antigen than when Rh incompatibility is responsible. In addition to the fact that most cases of AB-hemolytic disease are clinically mild, we are faced with a more complex serologic situation than is the case in HDN caused by sensitization to the Rh factor or even to other, more rarely involved, blood factors. In all other cases of HDN, detection in maternal serum of the antibody for a blood factor present in the infant's red cells represents prima facie evidence, whereas in HDN caused by AB incompatibility investigation of the maternal serum is hardly ever conclusive: physiologic or so-called "natural" isoagglutinins for the red cells of the infant are always present. Consequently, immunologic studies must be used to demonstrate quantitative and, if possible, qualitative changes in the antibody, especially the presence of "immune" or incomplete anti-A or anti-B. For the purpose of this presentation, complete isoagglutinins are defined as antibodies capable of agglutinating saline-suspended red cells, whereas incomplete isoagglutinins are antibodies not capable of doing so, but require the use of the indirect antiglobulin technique and/or papain-treated red cells for their detection.

The interpretation of results of prenatal and neonatal studies of maternal serum is further complicated by the fact that relatively high titers of incomplete ("immune") isoagglutinins may be found in ABO-heterospecific pregnancies that are not associated with HDN. Finally, neonatal diagnosis on the infant's blood present a further difficulty: in contrast to HDN caused by Rh and other isoantibodies, the direct antiglobulin (Coombs') test on the red cells of the infant is only rarely positive. In those rare cases the reaction is feeble, and requires special techniques for demonstration.

These circumstances have prompted many authors to investigate the possibility of finding some other method of diagnosis of the disease. Pertinent literature will be reviewed later.

The primary purpose of the present study was to test for the presence of the incompatible agglutinin in cord bloods of normal infants, as well as of infants suspected of or diagnosed as having HDN attributable to ABO incompatibility. We assumed that the situation in ABO incompatibility would be

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similar to that in Rh incompatibility, namely, that the offending antibody must traverse the placental barrier and hence appear in the infant's blood. In analogy to Rh sensitization, we assumed that incomplete antibodies would be probably associated with presence of disease. A preliminary report of this work was presented in 1956.[1]

MATERIAL AND METHODS

Most specimens tested were cord blood obtained at the time of delivery from presumably normal infants. The specimens were collected as clotted blood. Although heterospecific pregnancies were of primary interest, cord bloods from homospecific pregnancies were also tested. Some of the specimens, especially those of infants suspected of or diagnosed as having HDN, were venous blood.

ABO and Rh types were determined within 24 to 48 hours. Serum was separated from the red cells as soon as possible, and, if not tested immediately, was stored frozen at −20° C. Most tests were carried out with specimens not more than 1 to 2 days old. Blood groups and Rh types of the mothers were available from prenatal tests, and rechecked whenever indicated.

Isoagglutinins were tested for by volumetric titrations in which 0.1 ml. of the red cell suspension was added to 0.1 ml. of serum and of twofold serial dilutions of the serum. The following techniques were employed:

1. Mixtures of 2 per cent saline-suspended red cells and serum dilutions were centrifuged immediately (30 seconds in a small table centrifuge, commercially available as "Serofuge"[2]), and the readings recorded.

2. The tubes used in Technique 1 were shaken gently, incubated for 2 hours in a water bath at 37° C., read, and the results recorded.

3. Tubes not showing agglutination were converted into indirect antiglobulin tests.

4. In another set of titrations, papain-treated red cells[3] were added to the serum and serum dilutions and incubated at 37° C. for ½ hour.

5. Some specimens were also tested in a macromolecular medium, by using 2 per cent suspensions of red cells in 30 per cent bovine albumin and AB serum for dilution of the serum. Since this procedure did not produce agglutination as frequently as the other methods, it was omitted from the latter part of the study.

All tests were carried out with pooled fresh cells obtained from at least two individuals of groups A_1, B, and O. The latter cells were included as control for possible detection of nonspecific agglutinins.

RESULTS

Incompatible agglutinins were found in 6 of the 48 combinations of mother O-baby A, in 3 of the 10 combinations of mother B-baby AB, in 4 of the 27 combinations of mother O-baby B, and in one of the 11 combinations of mother A-baby AB. No instance of incompatible agglutinins was found among the other combinations. In 4 of the 14 cord bloods with incompatible agglutinins the antibody was complete. Clinical and hematologic examination of the 14 infants with incompatible agglutinins failed to disclose evidence of hemolytic disease.

In all 34 cases of hemolytic disease of the newborn, in which the presumptive diagnosis was first made on the basis of clinical and hematologic findings, incompatible incomplete agglutinins were present (Table I). In most instances both the antiglobulin technique and the test with enzyme-treated red cells gave positive results, but in 5 instances only the test with

papain-treated red cells, and in 4 only the antiglobulin test were positive. With either method the titers were low, rarely showing agglutination in serum dilutions higher than 1:2.

TABLE I. INCOMPATIBLE AGGLUTININS IN INFANTS WITH HDN CAUSED BY ABO INCOMPATIBILITY

BLOOD GROUP		NO. OF CASES	METHOD CAPABLE OF DETECTING					
			ANTI-A			ANTI-B		
MOTHER	INFANT		AGT	PAP.	AGT + PAP.	AGT	PAP.	AGT + PAP.
O	A	26	4	3	19			
B	AB	1		1				
O	B	5					1	4
A	AB	2						2
	Total	34	4	4	19		1	6

AGT = Antiglobulin technique.
PAP. = Papain-treated red cells.

In order to compare and evaluate the transplacental transfer of complete and incomplete anti-A and anti-B isoagglutinins, cord blood of homospecific pregnancies was also tested. Homospecific pregnancies are defined as those in which the mother does not possess isoagglutinins capable of agglutinating the red cells of her infant, whereas in heterospecific pregnancies mothers have such isoagglutinins. In Table II the findings in 106 homospecific and in 123 heterospecific pregnancies are summarized. In accord with previous investigations,[4-6] the highest percentage of infants with isoagglutinins demonstrable in cord blood was in those born to mothers of group O.

TABLE II. COMPLETE AND INCOMPLETE ISOAGGLUTININS IN CORD BLOOD OF NORMAL INFANTS

	BLOOD GROUP		NO. OF CASES	PRESENCE OF			
				ANTI-A		ANTI-B	
	MOTHER	INFANT		COMPLETE	INCOMPLETE	COMPLETE	INCOMPLETE
Homospecific pregnancies	O	O	29	26	3	26	2
	A	A	29			9	11
	A	O	18			8	0
	B	B	18	9	6		
	B	O	7	4	2		
	AB	A, B, AB	5				
		Total	106	39	11	43	13
Heterospecific pregnancies	O	A	48	1*	5*	34	8
	O	B	27	24	3	1*	3*
	A	B, AB	24				1*
	B	A, AB	24	2*	1*		
		Total	123	27	9	35	12

*Incompatible agglutinin.

The data shown in Table II lend themselves to an analysis of the relationship between the presence of isoagglutinins in mothers and their normal infants, exclusive of incompatible agglutinins. The results of this analysis are summarized in Table III. When anti-A was present in the mother, it was absent in less than 5 per cent of the compatible infants, whereas maternal

anti-B was absent in 21 per cent of infants not possessing the B antigen. This difference in behavior of anti-A and anti-B antibodies appears to be primarily the result of the less frequent passage of complete anti-B agglutinins as compared with anti-A, while transplacental transfer of incomplete antibodies is approximately the same for both antibodies.

TABLE III. RELATIONSHIP BETWEEN ISOAGGLUTININS IN MATERNAL AND CORD BLOODS IN NORMAL INFANTS*

ISOAGGLUTININ PRESENT IN MATERNAL SERUM	TYPE OF PREGNANCY	NO. OF CASES	ISOAGGLUTININ IN CORD SERUM							
			PRESENT						ABSENT	
			COMPLETE		INCOMPLETE		TOTAL			
			NO.	%	NO.	%	NO.	%	NO.	%
Anti-A	Homospecific	54	39	72.2	11	20.4	50	92.6	4	7.4
	Heterospecific	27	24	88.9	3	11.1	27	100.0	0	0
	All cases	81	63	77.6	14	17.6	77	95.2	4	4.8
Anti-B	Homospecific	76	43	56.6	13	17.1	56	73.7	20	26.3
	Heterospecific	48	34	70.9	8	16.6	42	87.5	6	12.5
	All cases	124	77	62.0	21	17.0	98	79.0	26	21.0

*Incompatible agglutinins excluded.

TABLE IV. REPORTS ON PRESENCE OF INCOMPATIBLE ISOAGGLUTININS IN CORD BLOOD

AUTHOR	YEAR	BLOOD GROUP		NO. OF CASES	METHOD USED*	TYPE OF ANTIBODY†	DISEASE IN INFANT
		MOTHER	INFANT				
Aubert et al.[7]	1945	B	A	1	?	?	Fatal HDN
Mitchell et al.[8]	1949	O	A	3	P, W	I	Fatal HDN
Wiener et al.[4]	1949	O	A	1	A	I	HDN
		O	B	1	A	I	HDN
Witebsky[9]	1949	B	A	1	AB	I	Fatal HDN
Robinson et al.[10]	1951	O	A	3	SA	I	HDN
Bryce et al.[11]	1952	O	A	1	AGT	I	HDN
Phansomboon and Pollack[12]	1952	O	A	2	W	I	Fatal HDN
Crawford et al.[13]	1953	O	A	8	AGT	I	HDN
Zuelzer and Kaplan[14]	1954	O	A	22	AGT	I	HDN
		O	A	1	S	C	HDN
		O	B	6	AGT	I	HDN
Seelen[15]	1954	O	A	2	S	C	Sl. jaundice
			Total	52			
Jakobowicz and Bryce[16]	1948	O	A	3	S	C	
		A	AB	1	S	C	
Wiener et al.[4]	1949	O	A	4	A	I	
		O	B	1	A	I	
		A	B	1	A	I	None
Zuelzer and Kaplan[17]	1954	O	A	3	AGT	I	
		O	A	1	AGT	I	
		O	A	1	S	C	
			Total	15			

*A = Acacia; AB = serum (group AB); AGT = indirect antiglobulin test; S = saline; P = plasma; SA = serum albumin; W = Witebsky neutralization.[18]
†C = Complete; I = incomplete.

DISCUSSION

Incompatible isoagglutinins in an infant with hemolytic disease were first reported in 1945 by Aubert and associates.[7] We have attempted to summarize additional reports from the literature in Table IV as they were available to us. Most of them were case reports or small series, until Zuelzer and Kaplan[14, 17]

reported a larger series in 1954. With the exception of 3 cases, the antibodies in the studies listed in the upper part of the table were incomplete; the methods used for their detection are shown in a separate column. The high incidence—7 out of 52—of fatal HDN is remarkable; it probably does not reflect the general clinical course in ABO incompatibility but rather the selected character of the series. Similarly, 19 of 34 cases listed by us in Table I were severe enough to necessitate replacement transfusion.

Table IV also contains data from the literature on presence of incompatible agglutinins in infants without any clinical disease. Significantly, 5 of 15, or one-third, of these antibodies were complete, i.e., demonstrable in saline medium. This incidence is similar to that of our own series (Table II).

On the basis of these findings of previous workers and of our own observations, the following remarks appear to be in order:

1. Incompatible agglutinins are present, as a rule, in the serum of infants affected with HDN caused by ABO incompatibility.

2. The antibodies are, as a rule, of the "incomplete" type, and hence require for their demonstration methods such as the antiglobulin technique or enzyme-treated red cells.

3. In our experience, the antiglobulin test or the technique employing papain-treated red cells proved capable of demonstrating the incompatible agglutinin in each of the 34 cases of hemolytic disease caused by AB incompatibility which we had an opportunity to investigate. Papain-treated red cells were sometimes successful in demonstrating the antibody when the indirect antiglobulin test failed to do so, and vice versa. Both methods should be used in each case. Our experiences with papain-treated cells appeared to confirm the statement by Spielmann[19] that the strongest and most rapid agglutination of group A red cells by anti-A agglutinins was seen with papain-treated red cells.

4. In our series, 14 of 123 infants in heterospecific pregnancies showed incompatible agglutinins *without* evidence of hemolytic disease. In 4, the agglutinins were detectable in saline, and hence presumably a "complete" antibody. Hence, while incompatible agglutinins were found in every case of HDN caused by AB incompatibility, this finding is not specific for the disease because they were also found in about 10 per cent of infants without demonstrable disease. This is not at all surprising since passage of anti-A or anti-B from mother to infant may occur without manifest clinical consequences. On the other hand, because of the frequently mild disease, the findings of incompatible isoagglutinins is to be considered as a warning, inviting closer observation and additional testing of the newborn.

5. Among the 34 infants with HDN caused by AB incompatibility, 27 involved incompatibility to the A antigen, and 7 to B. Several authors have previously recorded and commented on the greater frequency of maternal isosensitization to the A than to the B antigen as cause of HDN.[4-6] In our series of normal infants born from heterospecific pregnancies (Table II), transplacental transfer of incompatible anti-A was observed in 9 of 72 cases, and

of incompatible anti-B in 5 of 51 cases. These figures do not suggest that the placenta represents a greater barrier to incompatible anti-B than to incompatible anti-A.

As far as transplacental transfer of compatible agglutinins (Tables II and III) is concerned, the following observations were made: mothers of group O transmit compatible isoagglutinins (anti-A as well as anti-B) much more frequently to their infants than do mothers of group A. This holds true also for heterospecific pregnancies of O mothers with infants of group A or B. Mothers of group B transmit isoagglutinin anti-A almost as frequently as O mothers, but the antibody is more frequently incomplete. While, in general, mothers transmit anti-A more frequently to their infants than anti-B, this difference by itself does not seem to be capable of explaining the greater incidence of HDN caused by maternal isosensitization to A than to B. As a matter of fact, incomplete anti-B antibodies which most likely would be responsible for HDN appeared to traverse the placental barrier with about the same frequency as did incomplete anti-A. The more frequent appearance of anti-A than of anti-B in cord bloods may be merely the reflection of the fact that anti-A isoagglutinin reach higher titers in the maternal serum than do anti-B agglutinins. The explanation of the difference in the incidence of HDN caused by A and B isosensitization must be looked for in factors other than differences in transplacental transfer of the corresponding antibodies.

Our observations are in good accord with data obtained by other investigators on the frequency of transplacental transfer of isoagglutinins. Thus Polayes, Lederer, and Wiener[20] found anti-A in approximately 70 per cent and anti-B in 53 per cent of cord bloods of infants whose mothers possessed these antibodies. Since these antibodies were demonstrated with saline-suspended red cells, they may correspond to our category of "complete" antibodies of 77.6 per cent for anti-A and 62.0 per cent for anti-B (Table III). However, it must be kept in mind that the ability to agglutinate saline-suspended red cells by itself cannot be always taken as proving an antibody to be complete. One of us (I. D.[21]) showed that occasional serum specimens with high titers of incomplete Rh antibodies may agglutinate saline-suspended red cells when tested in undiluted serum or even in low dilution. Recently, Munk-Andersen[22] specifically pointed out that incomplete anti-A and anti-B agglutinins required even less colloid than Rh antibodies for bringing about agglutination of suitable red cells. In the present work the incomplete nature of isoagglutinins was considered as proved only if either saline-suspended red cells were not clumped at all or at a titer at least 2 dilutions lower than the titer obtained with the indirect antiglobulin test or with papain-treated red cells.

Using saline-suspended red cells, Tovey[23] found that in homospecific pregnancies anti-A was transmitted from mother to infant in 85 per cent and anti-B in 74 per cent.

On the basis of quantitative estimations of maternal and neonatal antibodies, he interpreted these findings as primarily reflecting higher maternal

titers of anti-A than of anti-B and not indicating differences in placental permeability for the two antibodies. That, in addition to "complete" isoagglutinins, cord bloods may contain "incomplete" anti-A and anti-B was demonstrated by Hirszfeld and associates[24] and by Wolf[25] by means of a dextran method, and by Wiener and Unger[6] who employed acacia or the indirect antiglobulin (Coombs') technique.

SUMMARY

1. Isoagglutinins anti-A or anti-B capable of reacting with red cells of the same ABO group as the infant (incompatible agglutinins) were demonstrated in the blood of each of 34 infants with HDN caused by ABO incompatibility.

2. The incompatible antibodies were of the "incomplete" type and could be best demonstrated with the indirect antiglobulin test or with papain-treated red cells.

3. Incompatible agglutinins were also found in approximately 12 per cent of cord bloods of normal infants born from heterospecific pregnancies; about one-third of these antibodies were of the "complete" type.

4. Data collected from the literature together with our own observations support the usefulness of tests for "incompatible" isoagglutinins in the neonatal diagnosis of HDN caused by ABO incompatibility.

5. From investigations of the transplacental transfer of anti-A and anti-B agglutinins it appears unlikely that differences in placental passage can account for the differences in the incidence of HDN caused by A and B incompatibility, respectively. The commonly higher titer of anti-A in the blood of the mother is probably sufficient to account for the difference.

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A COMPARATIVE STUDY OF THE ASHBY DIFFERENTIAL AGGLUTINATION, CARBON^{14} AND IRON^{59} METHODS FOR THE DETERMINATION OF RED CELL LIFESPAN

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ANEMIA not resulting from extravascular blood loss can only be due to a decrease in the rate of production of red cells, a decrease in the lifespan of the red cell, or a combination of these two factors. With the development in this laboratory of the radioactive iron method for the measurement of the rate of production of red cells, it was shown that in chronic leukemia the red cell production rate is usually normal or increased.[1] This implied that the red cell lifespan might be shortened. Studies of the lifespan of the red cell using C^{14}-labeled glycine showed that in patients with chronic myelogenous leukemia, and in some patients with chronic lymphatic leukemia, the red cell lifespan was shortened.[2] This is a direct method for the measurement of the red cell lifespan. At the time these studies were in progress, it became apparent that a re-evaluation of the Ashby differential agglutination technique for determining the lifespan of the red cell was necessary, since with this method normal cells are transfused to the patient or, on the other hand, red cells from a patient are transfused into a normal recipient.[3] In neither case is the red cell lifespan measured in its normal environment. It is entirely possible that the recipient will not treat the transfused cells in the same manner as his own. It is possible that an individual might produce red cells that are defective and have a short lifespan, while normal cells transfused to him would have a normal lifespan. For this reason, studies of the lifespan of red blood cells were undertaken using simultaneously the Ashby, the C^{14}-labeled glycine, and the Fe^{59} techniques.

METHODS

Thirteen patients were studied (see Appendix). Patients with blood groups A, AB, or B were transfused with red cells from group O normal donors, and the patients with group O and subgroup M red cells were transfused with subgroup N red cells.[3-7] The transfused red cells were counted at least once a week, according to the procedure of Sheets and associates.[4]

Plasma iron clearance and in vivo iron studies were done with Fe^{59}, as previously described.[1, 8] The Fe^{59} red cell uptake curves were followed for at least 150 days, except in patients R. F. and F. W.

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The lifespan of the red blood cells was measured with C^{14}-labeled glycine as described previously.[2]

The blood volume was determined with P^{32}-labeled red cells.[9]

RESULTS

The blood volumes are presented in Table I. Table II gives the plasma radioiron clearance data and summarizes the Fe^{59} in vivo studies.

TABLE I

PATIENT	BLOOD VOLUME		TOTAL RED CELL VOLUME		PLASMA VOLUME		HEMATOCRIT (PER CENT)	
	ML.	ML./KG.	ML.	ML./KG.	ML.	ML./KG.	PRCV*	PCV*
1. J. J.†								
2. J. S.	4,846	101.0	1,212	25.3	3,198	66.6	25	34
3. M. B.	3,671	61.2	1,597	26.6	1,909	31.8	44	47
4. F. T.	7,135	103.3	1,712	24.8	3,924	56.8	24	45
5. T. W.	3,558	61.1	1,281	22.0	2,277	39.1	36	
6. F. W.	4,419	53.7	1,282	15.6	3,137	38.1	29	
7. R. M.	4,260	67.0	1,747	27.5	2,513	39.5	41	
8. R. G.	5,040	67.5	2,117	28.3	2,873	38.5	42	
9. T. B.	5,591	77.9	2,208	30.8	2,963	47.1	40	43
10. R. F.	2,820	58.8	1,128	23.1	7,692	35.7	40	
11. A. F.	5,854	80.5	2,019	27.8	3,835	52.8	34	
12. L. F.	3,179	69.9	1,717	37.7	1,462	32.2	54	
13. T. A.	5,151	76.6	1,753	26.0	3,404	50.6	34	

*In cases in which the W.B.C. was elevated, both the packed red cell volume (PRCV) and the packed cell (red and white) volume (PCV) are indicated.

†No blood volume data available for this patient.

TABLE II

PATIENT	DATE	FE TURNOVER FOR HGB. FORMATION (MG./KG./DAY)	IN VIVO Fe^{59} STUDIES
1. J. J.*			
2. J. S.	2/15/52	1.69	Myeloid metaplasia
3. M. B.	2/8/54	0.17	Normal
4. F. T.	9/25/52	0.25	Splenectomy
5. T. W.	5/14/53	0.51	Normal
6. F. W.	4/22/53	0.11 0.14	High liver uptake†
7. R. M.	2/25/53	0.25	High liver uptake†
8. R. G.	5/6/52	0.59	Persistent spleen curve
9. T. B.	1/26/54	0.19	Persistent spleen curve
10. R. F.	4/3/52	0.30	Normal
11. A. F.	7/1/54	1.39	Secondary spleen curve
12. L. F.	11/10/52	0.57	(Not done)
13. T. A.*			

*Fe^{59} studies not carried out.

†Liver uptake: F. W., 2.77 mg. Fe per gram of tissue; R. M., 8 mg. Fe per gram of tissue, measured from liver biopsies.

Ashby Studies.—When the rate of decrease of transfused cells was predominantly linear, the linear segment of the data was fitted to a straight line by the least squares method. The red cell lifespan and the maximum concentration of inagglutinable cells were obtained by extrapolation (see Figs. 1 and 2). In the 10 patients in this group the red cell lifespan varied from 98 to 136 days.

The other 3 patients, A. F. (Fig. 3), L. F. (Fig. 4), and T. A. (Fig. 5), showed an exponential rate of disappearance of donor cells (Table III). The

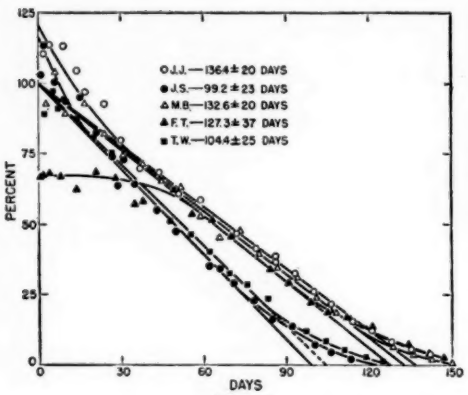

Fig. 1.

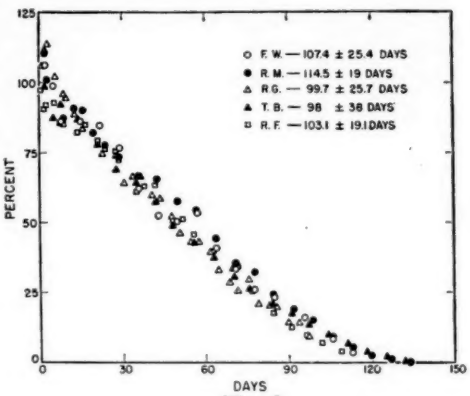

Fig. 2.

Fig. 1.—Ashby differential agglutination data for Patients J.J., J.S., M.B., F.T., and T.W.

Fig. 2.—Ashby differential agglutination data for Patients F.W., R.M., R.G., T.B., and R.F.

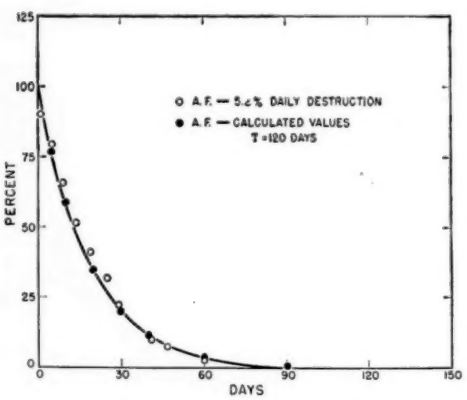

Fig. 3.

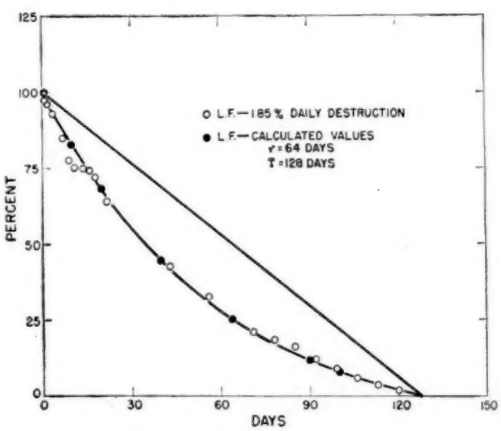

Fig. 4.

Fig. 3.—Ashby differential agglutination data for Patient A.F.
Fig. 4.—Ashby differential agglutination data for Patient L.F.

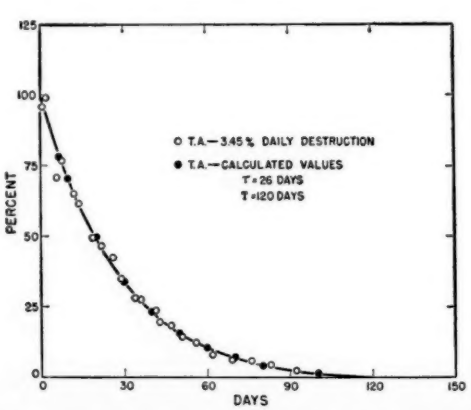

Fig. 5.—Ashby differential agglutination data for Patient T.A.

theoretical values were calculated according to Sheets and associates,[4] assuming a random destruction at a constant rate plus normal aging:

$$N = N_o \left(1 - \frac{t}{T}\right) e^{-\frac{0.693\ t}{\tau}}$$

where N = Concentration of donor cells at time t after transfusion; N_o = Concentration of donor cells at $t = 0$; T = Time after transfusion when all the donor's cells would have been lost by senescence; and τ = Time after transfusion when N is half the number of cells which would have been present if loss were due only to senescence.

That the exponential disappearance shown by these 3 patients can be explained as a combination of random destruction and normal aging is shown by the close agreement of the observed values with those calculated from this equation.[4]

TABLE III

PATIENT	SEX	DISEASE	RED CELL LIFESPAN (DAYS)			RATE OF RED CELL FORMATION (IN % RENEWAL/DAY FROM FE^{59} STUDIES)
			ASHBY	C^{14}	FE^{59}	
1. J. J. (Figs. 1, 9)	M	Chronic myelogenous leukemia (?) and tuberculosis	136.4	142	142	
2. J. S. (Figs. 1, 6)	F	Myeloid metaplasia	99.2	115		6.7
3. M. B. (Figs. 1, 7)	F	Chronic myelogenous leukemia	132.6	114		0.67
4. F. T. (Figs. 1, 8)	M	Chronic myelogenous leukemia	127.3	118	120	1.01
5. T. W. (Figs. 1, 16)	M	Anemia of undetermined etiology	104.4	76	63	2.31
6. F. W. (Figs. 2, 14)	M	Aplastic anemia; hemochromatosis	107.4	87	80	0.71
7. R. M. (Figs. 2, 15)	M	Diabetes mellitus; hemochromatosis	114.5	113		0.91
8. R. G. (Figs. 2, 12)	M	Giant follicle lymphoblastoma	99.7	105	99	2.08
9. T. B. (Figs. 2, 10)	M	Chronic lymphatic leukemia	98.0	101	88	0.62
10. R. F. (Figs. 2, 13)	F	Giant follicle lymphoblastoma	103.1	97		1.30
11. A. F. (Figs. 3, 17)	M	Chronic lymphatic leukemia	5.2% daily destruction	31		5.7
12. L. F. (Figs. 4, 11)	F	Secondary polycythemia	1.85% daily destruction	76	80	1.51
13. T. A. (Fig. 5)	M	Chronic lymphatic leukemia	3.45% daily destruction			

One patient (F. T.) with chronic myelocytic leukemia (Fig. 1) showed no destruction for at least 40 days. This patient had had a splenectomy one year earlier and had been given a therapeutic dose of 4 mc. of P^{32} intravenously two days after the transfusion of Ashby cells. By reducing the rate of production of red cells and also the blood volume, P^{32} probably effected a temporary net concentration of donor cells.

The variation from a straight line in the disappearance of transfused cells in the early days following the transfusion has been attributed to the difference between the rate of breakdown of the donor's and recipient's red cells.[5] For this reason, the same donor was used several times for several recipients.

TABLE IV

DONOR	RECIPIENT	AGING TIME (DAYS)	RED CELL LIFE (DAYS)	INITIAL VALUE (PER CENT)	CALCULATED RED CELL LIFE DONOR (DAYS)
R. W	T. A.		R. D.		
	T. W.	79.4	104.4	100	104.4
	M. B	112.6	132.6	100	132.6
T. M.	F. W.	82.0	107.4	110	118.1
	J. J.	116.4	136.4	120	163.7
F. G.	R. F.	84.0	103.1	92	94.9
	L. F.		R. D.		

R. D. = random destruction.

The results are tabulated in Table IV. The red cell life of the donor cells may be calculated in the following way[5]:

Red cell life donor $= N_o$ (k),

where $k = \frac{1}{\text{slope (straight segment of Ashby curve)}}$.

For donor R. W. (Table IV) there was no such initial variation. The red cell life of the donor then should be the same as that of the recipient, but the 2 recipients had different red cell lifespans. With the donor T. M. there was a different initial destruction rate in the two recipients, and the lives of the same donor cells calculated on this basis are different. It is possible then that the variation from a straight line in the first part of the curve is due principally to the fragility of the cells toward transfusion manipulations.

Evans and co-workers[5] state that the deviation from linearity in the last part of the Ashby differential agglutination curves can be explained by assuming that normal cells "undergo a period of aging" and that "aged cells are broken down by a random, first-order process." Table IV shows that the "aging times" of cells from the same donor vary in different recipients.

TABLE V. COMPARISON OF DURATION OF TAIL PORTION OF ASHBY CURVE WITH C^{14} CURVE

PATIENT	ASHBY (DAYS)	C^{14} (DAYS)
1. J. J.	± 20	± 20
2. J. S.	± 23	± 23
3. M. B.	± 20	± 19
4. F. T.	± 37	± 43
6. F. W.	± 25	± 23
7. R. M.	± 19	± 35
8. R. G.	± 26	± 29
9. T. B.	± 38	± 37
10. R. F.	± 19	± 18

The tail portion of the curve calculated according to Evans and associates[5] shows an average life of the worn cells ranging between ±19 to ±38 days, in agreement with that found by Evans and co-workers (Table V).

C^{14}- and Fe^{59}-Labeled Cell Method.—Ten of the 12 patients studied showed a plateau of Fe^{59} or C^{14} specific activity (Figs. 6-10, 12-16). The mean of the plateau was considered as 100 per cent.

In patients L. F. and A. F. (Figs. 11 and 17), whose graphs showed no plateaus, the highest values were considered as 100 per cent. In these two patients the red cell lifespan as measured with carbon^{14}-labeled glycine represents an upper limit. It is quite likely the true mean red cell lifespan is shorter. The effect of continual synthesis of labeled hemoglobin from persisting labeled glycine tends to make the slope of the curve of hemoglobin carbon^{14} content smaller than would occur if there were not a continual synthesis. It is relatively easy to show that when the red cell lifespan is finite and normal this tends to overestimate the red cell lifespan only slightly.[10] Mathematical methods for the correction of the observed carbon^{14} content of hemoglobin, when the pattern is one of a very short red cell lifespan and random destruction, have not been devised. It can only be stated that the actual red cell lifespan will be less than that calculated from the data and, at present, of an unknown magnitude.

The red cell lifespans as determined by C^{14} and Fe^{59} are given in Table III. They are obtained by taking the time between the ascending and descending parts of the curves at half the distance between the upper and lower plateaus. The descending part of the curve in the C^{14} results, which corresponds to the random destruction of worn cells, might be compared with the exponential destruction of the worn Ashby cells (Table IV). Six patients (T. W., F. W., T. B., A. F., L. F., and T. A.) had short red cell lifespans as determined either by the Ashby technique or by both the Ashby and C^{14} methods. Of the 10 patients on whom in vivo Fe^{59} studies were done (see Table II), 4 had abnormal spleen curves. In 3 there was either a rise in the counting rate over the spleen followed by a fall and then a secondary rise, or there was a rise with no fall. These types of spleen curves have been interpreted as indicating premature destruction of red cells in the spleen.[11] Because of the large reutilization of iron, the curves obtained using the Fe^{59} method do not have the sharp fall associated with the C^{14} or N^{15} studies, and analysis of the lifespan is not as satisfactory. Since it cannot be assumed that any of these patients was in a steady state with regard to red cell production and destruction, calculation of the red cell lifespan from the red cell iron turnover is misleading.

Comparative Study of the Red Cell Lifespan With the Three Methods.—For comparison, the Ashby, C^{14}, and Fe^{59} results were plotted on the same graph. The Ashby data were corrected for daily destruction in the following manner: If the red cell life is T, at N days after the beginning of the experiment, the plotted inagglutinable cells are equal to:

$$\frac{100\ (T-N)}{(\%\ \text{inagglutinable cells at N})\ T}.$$

When the red cells produced in the patient are normal and destroyed only through senescense, the results should agree in all three methods. They agree in 9 patients within the limits of error (Table III), and when the rate of production is sufficient, the total red cell volume will be normal.

Patient T. W. (Fig. 16) showed a normal red cell life with the Ashby technique and a finite but shortened red cell life with C^{14} and Fe^{59}. Here the

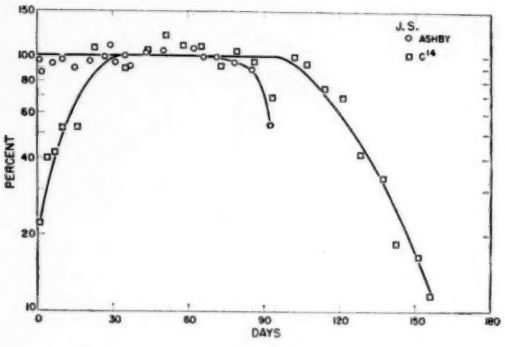

Fig. 6.

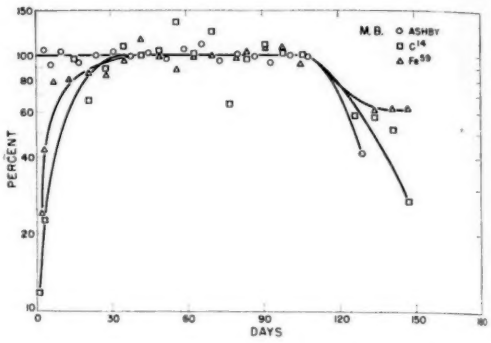

Fig. 7.

Fig. 6.—Red cell lifespan curves by the Ashby and C^{14} methods for Patient J.S.
Fig. 7.—Red cell lifespan curves by the Ashby, C^{14}, and Fe^{59} methods for Patient M.B.

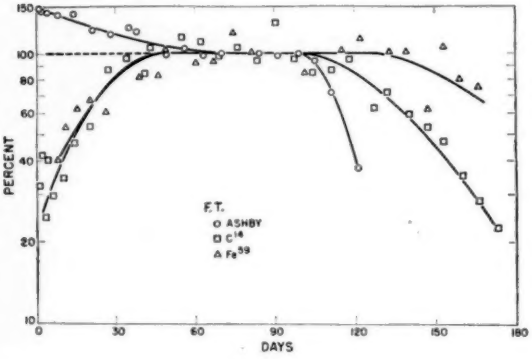

Fig. 8.

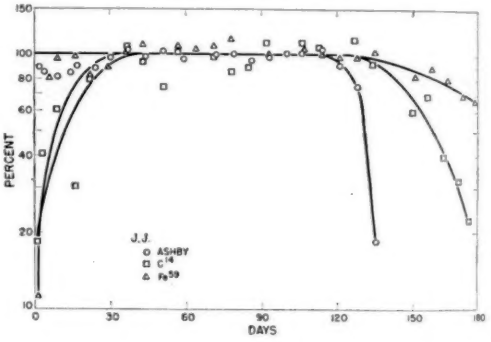

Fig. 9.

Fig. 8.—Red cell lifespan curves by the Ashby, C^{14}, and Fe^{59} methods for Patient F.T.
Fig. 9.—Red cell lifespan curves by the Ashby, C^{14}, and Fe^{59} methods for Patient J.J.

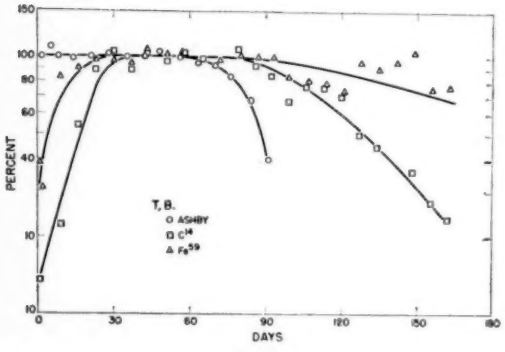

Fig. 10.

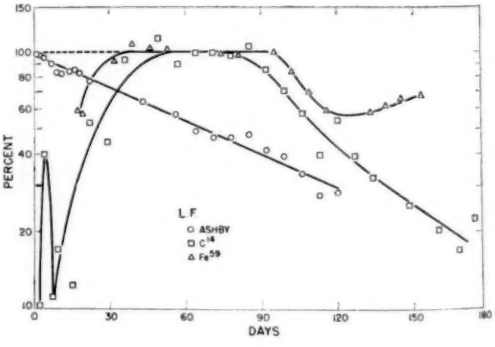

Fig. 11.

Fig. 10.—Red cell lifespan curves by the Ashby, C^{14}, and Fe^{59} methods for Patient T.B.
Fig. 11.—Red cell lifespan curves by the Ashby, C^{14}, and Fe^{59} methods for Patient L.F.

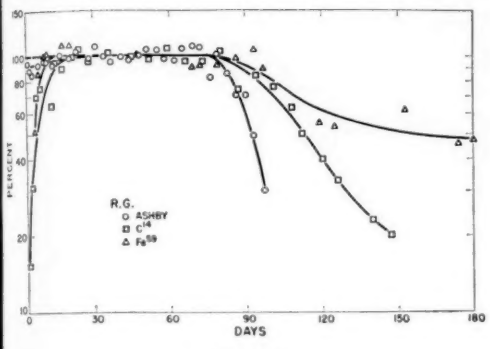

Fig. 12.

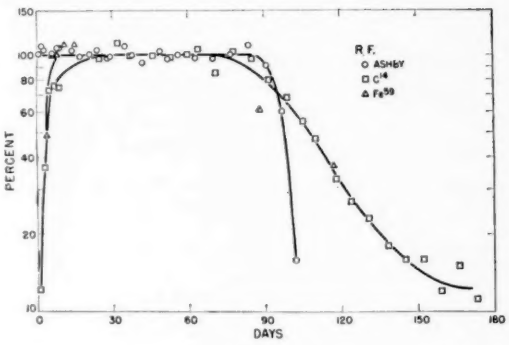

Fig. 13.

Fig. 12.—Red cell lifespan curves by the Ashby, C^{14}, and Fe^{59} methods for Patient R.G.
Fig. 13.—Red cell lifespan curves by the Ashby, C^{14}, and Fe^{59} methods for Patient R.F.

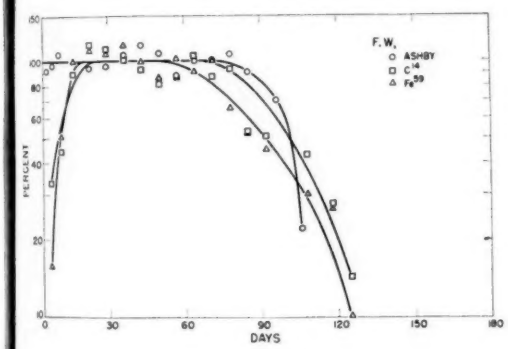

Fig. 14.

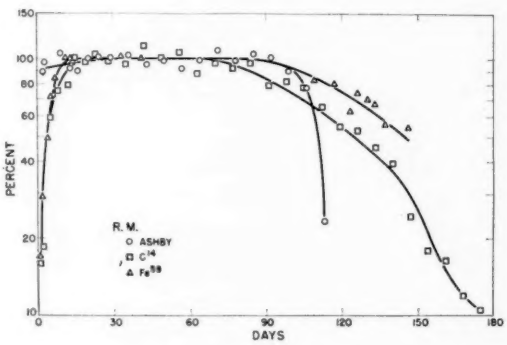

Fig. 15.

Fig. 14.—Red cell lifespan curves by the Ashby, C^{14}, and Fe^{59} methods for Patient F.W.
Fig. 15.—Red cell lifespan curves by the Ashby, C^{14}, and Fe^{59} methods for Patient R.M.

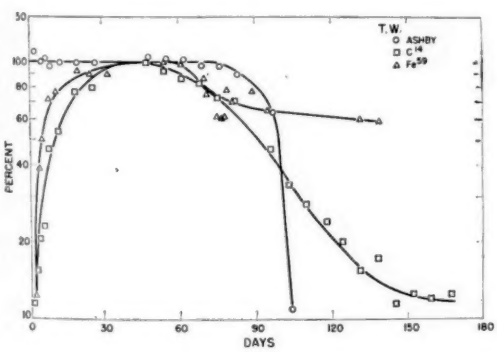

Fig. 16.

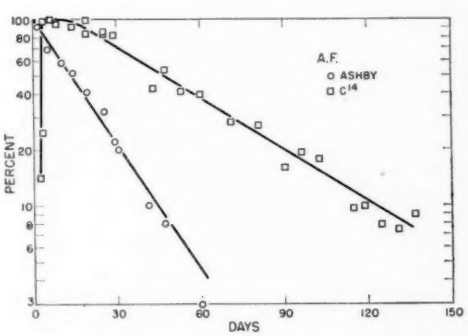

Fig. 17.

Fig. 16.—Red cell lifespan curves by the Ashby, C^{14}, and Fe^{59} methods for Patient T.W.
Fig. 17.—Red cell lifespan curves by the Ashby and C^{14} methods for Patient A.F.

quality of the cells must have been deficient, as normal cells survive normally in the patient while the patient's own cells have a finite but shortened lifespan. The rate of production as measured with Fe^{59} was elevated, indicating an increased production of red cells which partially compensated for their short lifespan, although the patient was anemic by blood volume standards.

Patient L. F. (Fig. 11) showed random destruction with a rate of 1.85 per cent per day (normal, 0.85 per cent) with the Ashby technique and a short but apparently finite red cell life of 76 days for C^{14} and 80 days for Fe^{59}. The patient's cells might have had a normal red cell life if given to a normal subject, but in the patient they undergo a random destruction as shown by the Ashby technique. The high plasma iron turnover associated with red cell formation indicates an increased production rate that compensates for the increased destruction and keeps the total red cell volume at a slightly polycythemic level.

In Patient F. W. (Fig. 14) the red cell life obtained by the Ashby technique was normal (107 days) and somewhat longer than the red cell life as measured by the two other methods (87 days for C^{14} and 80 days for Fe^{59}). The rate of formation of red cells as measured by Fe^{59} was low, indicating a decreased production of red cells which might have been slightly deficient in some as yet unknown manner as compared to normal red cells. Both these factors would explain the anemia of this patient, who needed frequent transfusions.

Patient F. T. (Fig. 8), who had been splenectomized, had a very long red cell life by all three methods, and all three methods yielded comparable values.

Red Cell Lifespan in Disease.—

Chronic myelogenous leukemia: In previous studies it was reported that the red cell lifespan was shortened in chronic myelogenous leukemia but that the lifespan was finite.[2] Two patients with chronic myelogenous leukemia in this study had normal red cell lifespans. In one (F. T.) this was following a splenectomy and is in agreement with Ragnar Berlin's[12] observations on the lengthening of the red cell lifespan in chronic myelogenous leukemia following splenectomy. In the other patient (M. B.) this was very early in the course of her disease, presumably within 6 months of the onset of leukemia. Further observations at a later time would be of interest to determine if and when shortening of red cell lifespan does develop.

Chronic lymphatic leukemia: In agreement with previous studies, the lifespan of the red cell in chronic lymphatic leukemia was found to be either normal or very short with a pattern of random destruction.[2] In patient A. F. conversion to a normal red cell lifespan was observed following splenectomy.

Giant follicle lymphoblastoma: Both patients with giant follicle lymphoblastoma (R. G. and R. F.) had a normal red cell lifespan.

Unfortunately these studies do not differentiate in those patients with a short red cell lifespan—either with random destruction or with a shortened but finite red cell lifespan—the nature of the defect leading to a decrease in the lifespan of the cell. In Patient A. F. the conversion from a short red cell lifespan with random destruction to a normal cell life following a splenectomy strongly suggests the presence of an extrinsic factor arising in or mediated through the spleen.[13] In fact, on theoretical grounds, in the absence of hemor-

rhage, the pattern of random destruction is best explained by some extracorpuscular factor, and the shortened but finite red cell lifespan by some intracorpuscular defect. These studies are not conclusive in this regard, however.

CONCLUSIONS

1. In 8 patients the red cell lifespan was normal as determined by glycine labeled with C^{14} and by the Fe^{59} and Ashby techniques. The results of all three methods were in good agreement.

2. The Ashby technique is of value in interpreting the data shown by the C^{14} and Fe^{59} methods.

3. In the 2 cases presumably with hemolysis due to extracorpuscular factors, all three methods showed a very short cell life.

4. In 2 cases (T. W. and F. W.) the quality of the cells produced was defective, and the Ashby technique showed a normal red cell life while the C^{14} and Fe^{59} methods showed a shorter red cell life.

APPENDIX

CASE 1.—J. J., a 65-year-old white man, was first seen here in September, 1953. A diagnosis of pulmonary tuberculosis was made in 1951, and the patient was treated. In November, 1952, elevation of the white cell count and an abnormal differential suggested chronic myelogenous leukemia. The patient was placed on Myleran in 1954, and the white count fell to normal with a normal differential. In December, 1954, a lobectomy was performed because of cavitation in the left upper lobe. The patient expired 12 hours after surgery. At autopsy no evidence of myelogenous leukemia could be found. Whether this should be viewed as a leukemoid response to tuberculosis or whether it represented an excellent response to Myleran with a conversion of the bone marrow to normal is problematical. The absence of histologic evidence of leukemia in spleen, liver, and bone marrow made the diagnosis difficult.

CASE 2.—J. S., a 51-year-old white woman, was first seen in February, 1952. She gave a history of an onset of arthritis in 1949. In September, 1950, the patient was told a blood count showed leukemia. In 1951 the spleen became palpable. Physical examination disclosed a thin, fairly developed woman, with deforming rheumatoid arthritis of the elbows, wrists, fingers, knees, and ankles. The blood count showed R.B.C. 3.53 million; hemoglobin 7.8 Gm.; platelets 400,000; and W.B.C. 140,000, with a differential compatible with that of myelogenous leukemia. Iron studies showed an excessive iron turnover, only part of which could be attributed to excessive turnover through the red cells. The serial spleen site counting rate curve indicated a primary path through the spleen, with a subnormal rise in the marrow site counting rate, suggesting splenic extramedullary erythrogenesis. The decline in the counting rate over the spleen simultaneously with the ascending radioactivity in the red cells is believed to be confirmatory of splenic erythropoiesis. As a result of liver, spleen, and bone marrow biopsies and the radioiron studies, a diagnosis of myeloid metaplasia was made. The patient has been treated throughout with cortisone.

CASE 3.—M. B., a 38-year-old white woman, was first seen here in February, 1954, with chronic myelogenous leukemia of approximately 4 months' duration. At the time these studies were carried out, the liver and spleen were not palpable. The blood count was 4.63 million, with 14.6 Gm. of hemoglobin, platelets 530,000, and white count 81,500, with a differential compatible with that of chronic myelogenous leukemia. This is the first patient with chronic myelogenous leukemia studied in this laboratory to have a normal red cell lifespan in the presence of a spleen (see Case 4, F. T., for results in splenectomy). It is probable that this woman was studied so early in the course of the disease that alteration in the lifespan of the red cell was not yet evident

CASE 4.—F. T., a 36-year-old white man, was first seen here in May, 1952, with chronic myelogenous leukemia of one year's duration. A splenectomy had been done in 1951, fol-

lowed by x-ray therapy. At the time he was first seen here the blood count was 3.58 million; hemoglobin 8.5 Gm.; platelets 1,880,000, and W.B.C. 331,000.

CASE 5.—T. W., a 67-year-old white man, was first seen here in January, 1953, with the principal complaint of weakness and fatigue for the preceding 18 months. He was referred here with a diagnosis of anemia of unknown etiology. At the time of his first visit the blood count showed R.B.C. 3.94 million; hemoglobin 11.8 Gm.; and W.B.C. 5,200 with a normal differential. The blood volume determination showed 22 ml. per kilogram of red cells and a plasma volume of 39.1 ml. per kilogram. This was a moderate anemia, but despite extensive investigation the cause has not yet been established.

CASE 6.—F. W., a 74-year-old white man, was seen at this laboratory in March, 1953, with a history of anemia. A diagnosis of carcinoma of the prostate was made following a transurethral resection in 1952. Following surgery, the patient was found to be anemic and did not respond to therapy. Radioactive iron studies showed that this anemia was due to a failure in red cell production. This man required frequent transfusions to maintain the blood count at approximately 2.5 to 3.5 million, with 6.8 to 9.3 Gm. of hemoglobin. The white cell count was never remarkable. Biopsy of the bone marrow showed hypoplasia. The liver biopsy showed hemosiderin pigmentation characteristic of hemochromatosis, probably secondary to multiple transfusions.

CASE 7.—R. M., a 45-year-old white man, was first seen here in October, 1952, with a diagnosis of hemochromatosis and diabetes. At the time these studies were done, the patient had the following blood count: R.B.C. 4.92 million; hemoglobin 13.8 Gm.; and W.B.C. 6,200, with a differential showing 39 per cent segmented and 2 per cent nonsegmented cells, 2 per cent eosinophils, 51 per cent lymphocytes, and 5 per cent monocytes. In vivo iron studies showed a greater than normal amount of iron disappearing from the plasma to liver. A sample of liver tissue contained 8 mg. of iron per gram of liver tissue. Urinalysis showed 3+ sugar. Pigmentation was found on the backs of the arms, legs, periumbilical area, and the axilla. The liver was hard and easily felt almost 2 cm. above the umbilicus. The spleen was not palpable.

CASE 8.—R. G., a 52-year-old white man, was first seen in October, 1949. The patient had noticed right cervical lymphadenopathy in 1944, and a biopsy revealed giant follicle lymphoblastoma. He was treated with x-radiation to the right inguinal and right axillary regions at approximately 6- to 12-month intervals. The last x-ray therapy before being seen here was given a month previously. On physical examination it was noted that there were ½ to 1 cm. nodes in both axillae and in the right inguinal region. No cervical nodes were palpable. The liver was palpable to the level of the iliac crest. The blood count showed R.B.C. 3.61 million; hemoglobin 11 Gm.; and W.B.C. 7,500, with a normal differential. The red cell lifespan studies were started in May, 1952. At this time there was only one node palpable, in the right posterior cervical triangle. The liver and spleen were not palpable. The blood count then showed R.B.C. 4.09 million; hemoglobin 11.3 Gm.; and W.B.C. 6,150, with 45 per cent lymphocytes.

CASE 9.—T. B., a 66-year-old white man, was first seen here in June, 1952, with a known chronic lymphatic leukemia of 10 years' duration. At the time these studies were done the spleen was palpable 2 cm. below the costal margin, the liver edge was palpable about 1 to 2 cm. on deep inspiration, and there was one large (2 to 3 cm.) node in the right axilla with smaller lymph nodes palpable in the left axilla and inguinal regions. The blood count done at this time showed: R.B.C. 3.82 million; hemoglobin 12.8 Gm.; platelets 140,000; and W.B.C. 97,000, with 99 per cent lymphocytes and 1 per cent polymorphonuclear leukocytes.

CASE 10.—R. F., a 26-year-old white woman, was first seen here in March, 1952, at which time the patient gave a history of a mass in the left breast of approximately one month's duration. On biopsy, this was found to be a giant follicle lymphoblastoma. There was also a broad tender mass in the upper abdomen which biopsy showed to be a giant follicle lymphoblastoma. The spleen was enlarged. The blood count at this time was as follows: R.B.C. 3.94 million; hemoglobin 11.7 Gm.; and W.B.C. 10,800, with 49 per cent lymphocytes.

CASE 11.—A. F., a 53-year-old white man, had chronic lymphatic leukemia of 2 years' duration. Because of the fact that he had had several episodes of chills and fever which

responded to antibiotic therapy and because of the fact that he had a large palpable spleen with a relatively normal white cell count at the time of his first visit here in the spring of 1954, a biopsy of the spleen was done, as well as a sternal marrow biopsy. The spleen tissue showed small, granulomatous lesions, the etiology of which is still unknown despite extensive serologic and bacteriologic investigations. A splenectomy was done, following which a normal red cell lifespan was observed, as measured with C^{14}.

CASE 12.—L. F., a 47-year-old woman, was referred to us in October, 1952, with a diagnosis of pulmonary fibrosis and secondary polycythemia. The patient had a history of shortness of breath for 10 years, and the pulmonary fibrosis was discovered during an episode of influenza in 1949. An elevated red blood cell count had been found 2 months before her first visit here, and a 500 ml. phlebotomy had been performed. The patient reported a 30-pound weight loss in the preceding 3 years. On physical examination, redness of the face, hands, and mucous membranes was noted. Decreased resonance of the right middle lobe of the lungs was noted, with increased resonance elsewhere. There was an increased heart rate, and the blood pressure was 170/120 RAR.* The spleen and liver were not palpaple. The initial blood count was R.B.C. 7.16; hemoglobin 19.0 Gm.; and W.B.C. 15,500, with a normal differential count. Blood oxygen saturation was 75 per cent. The turnover of iron for red cell production was increased.

CASE 13.—T. A., a 53-year-old white man, had chronic lymphatic leukemia. He was first seen here in November, 1949. Onset of the disease occurred in the summer of 1949, when enlargement of the lymph nodes was observed. Biopsy showed lymphoid hyperplasia suggestive of leukemia. His first blood count here showed: R.B.C. 3.78 million; hemoglobin 13 Gm.; platelets 260,000; and W.B.C. 12,100, with a normal differential. A bone marrow puncture done at that time showed 46.2 per cent to be of the lymphocytic series. The studies of red cell life were done in 1952 when the patient had a red count of 3 million and a differential white count varying from 44 to 72 per cent lymphocytes. The spleen was enlarged to the umbilicus. The liver was not palpable. From 1949 to this time the patient had received therapy with x-ray and P^{32}.

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*RAR = right arm recumbent.

STUDIES OF PLATELET SURVIVAL BY TAGGING IN VIVO WITH P^{32}

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INTRODUCTION

WITH isotopic techniques it has become possible to study the survival of normal platelets in normal subjects. Two basic methods have been used for platelet tagging: in one the platelets are tagged in vitro by incubation with the isotope; and in the other the isotope is injected into the subject whose platelets are then tagged in vivo. With the in vitro method, considerable incubation, manipulation, and washing of the platelets are involved and their survival has usually been short, sometimes only a few hours.[1-7] Where the in vivo method of tagging has been employed, the survival in general has been longer.[3, 8, 9] However, in all but a few[8, 9] of the reported experiments, the platelets have been separated and washed at least one time before transfusion. There is considerable evidence that sedimenting of platelets by centrifugation may lead to damage which may result in shortened survival.

Leeksma and Cohen[9, 10] have reported a method of tagging platelets in vivo which avoids platelet manipulation and transfusion. The platelets are tagged by an intramuscular injection of diisopropylfluorophosphonate containing P^{32} (DFP^{32}). Several objections to this method may be raised. First, the DFP^{32} may form a depot in the recipient animal, and tagging may continue over a period of several days rather than for several hours, as is assumed. This would give a falsely long survival. Second, this method when applied to white blood cells gives a survival of only a few hours, whereas with most other techniques the white cells survive for many days or weeks. This suggests the possibility of elution of the absorbed DFP^{32}. Third, there is no proof that the absorbed DFP^{32} does not alter the platelets and therefore alter their survival.

We have developed a technique of tagging platelets in vivo and transfusing them with a minimum of manipulation and no incubation, centrifugation, or washing. Since a transfusion is involved, this method can be used to study the survival of isogenous platelets only; it cannot be applied to the survival of autogenous platelets. However, it has the advantage that the P^{32} is actually part of the platelet structure rather than adsorbed onto the platelet surface or taken up by dynamic metabolic interchange with the plasma phosphates.

METHODS

In brief, the method involves the injection of a donor with P^{32}, waiting an appropriate time while a population of radioactive platelets is produced, and then transfusing the donor's whole blood into a recipient. After the transfusion, samples of platelets are isolated from

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the recipient's blood and their radioactivity is measured. This is repeated at appropriate intervals. The diminishing level of radioactivity in the isolated platelets reflects the disappearance of the transfused platelets.

In the animal work, normal dogs weighing approximately 12 Kg. were used as platelet donors and platelet recipients. The donor animals received 1 mc. of P^{32} intravenously. In the human work, patients with polycythemia vera were the donors and normal volunteers were the recipients. The human donors received 3 to 5 mc. of P^{32} intravenously. One week after the P^{32} injection, blood was drawn into plastic bags with disodium ethylenediaminetetraacetate (EDTA) as the anticoagulant. The volume taken was 500 ml. from the humans and 300 ml. from the dogs. In the animal work, the recipient dog was prepared by a phlebotomy of 300 ml.. This was immediately followed by transfusion with the radioactive blood. At no time in either the human or animal experiments did the interval between the drawing of the radioactive blood and its transfusion exceed 30 minutes.

Platelet survival was determined by drawing samples of blood from the recipients at various intervals. A two-syringe technique was used, all glassware was siliconized, and EDTA was used as the anticoagulant. The platelets were separated by differential centrifugation, and the number of platelets was counted by phase microscopy.[11] The platelets were washed repeatedly and were plated onto an aluminum planchet. The radioactivity was counted by means of a thin-window Geiger tube. To overcome the problem of physical decay of P^{32}, all radioactive samples from each experiment were counted at the same time.

Platelet-free plasma was obtained from samples of whole blood drawn by the two-syringe technique with oxalate as the anticoagulant. The blood was centrifuged at 3,000 r.p.m. for 2 hours, and a volume of the supernatant plasma was plated onto a planchet and counted in the same manner as the platelets.

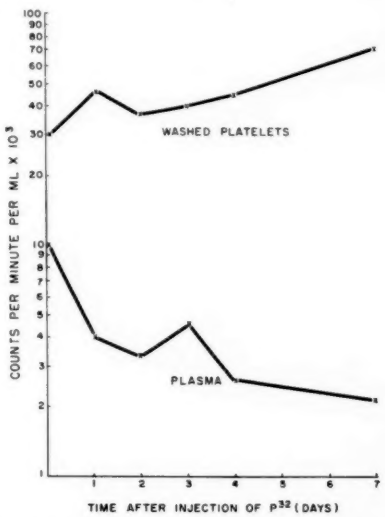

Fig. 1.—The incorporation of injected P^{32} into the circulating platelets of a patient with polycythemia vera (Subject A).

RESULTS AND DISCUSSION

Immediately after the injection of P^{32}, the radioactivity of the plasma is high (Fig. 1), but it rapidly falls to a low level and thereafter gradually disappears. During this disappearance the radioactivity of the platelets climbs to a level which is many times that of the ambient plasma, then gradually subsides. At this peak of activity the platelets are harvested.

The activity which appears in the platelets separated from the first sample (drawn 30 minutes after injection) may represent P^{32} adsorbed onto the surface

of the platelet. Most of the injected P^{32} probably enters the phosphorus pool of the body, and megakaryocytes draw upon this material for the phosphorus that is used in the formation of new platelets. The appearance of new platelets containing P^{32} in their stroma probably accounts for the rising platelet activity during the first 7 days, in the face of a falling plasma activity. The ideal time for phlebotomy appears to be 7 days after the injection, since the platelet activity at that time is at its highest point and plasma activity is low.

A second example of plasma and platelet radioactivity in a human polycythemic subject after injection of P^{32} is shown in Fig. 2. Here the ideal time for phlebotomy would have been the sixth day.

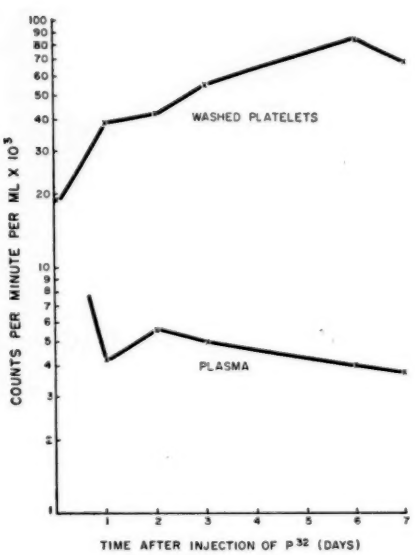

Fig. 2.

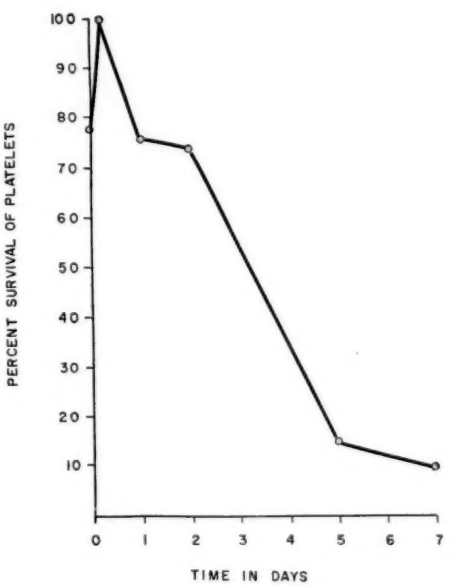

Fig. 3.

Fig. 2.—The incorporation of injected P^{32} into the circulating platelets of a patient with polycythemia vera (Subject B).

Fig. 3.—The survival of transfused radioactive platelets in recipient Dog 1.

Fig. 3 shows the typical survival of radioactive platelets in a normal dog recipient. The curve of decay appears to be exponential, with a half-life of about 3 days. It reached the 10 per cent level on the seventh day. On the day of the transfusion the 30-minute sample showed much less activity than the 3-hour sample. This rise in platelet radioactivity during the first hour post transfusion was a regular occurrence. It may have been due to transient sequestration of the transfused platelets. An alternative explanation is that P^{32} from the donor's plasma was transferred to the recipient's platelets.

To test this possibility, a dog was given intravenously 1 mc. of P^{32}. One week later the animal was phlebotomized. Platelet-free plasma was obtained from the blood and transfused into a recipient dog. Thereafter no significant radioactivity could be demonstrated on the recipient's platelets, which indicated that there was no significant transfer of P^{32} from transfused plasma to the recipient's platelets.

It seems more likely, therefore, that the rise in platelet radioactivity after the 30-minute sample was because of the appearance of platelets which had been briefly sequestrated following their transfusion.

To prove that the curves we obtain are truly a measure of platelet survival and not due to artefact or to P^{32} reutilization, an experiment was carried out in which nonviable platelets were transfused. A donor animal received

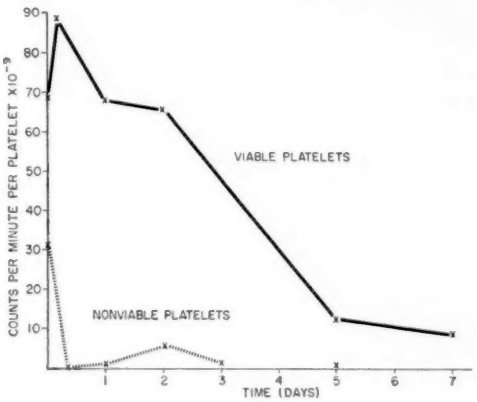

Fig. 4.—The survival of transfused radioactive platelets. The curve representing loss of viable platelets is the same as Fig. 3, shown for comparison. The nonviable platelets had been stored in glass.

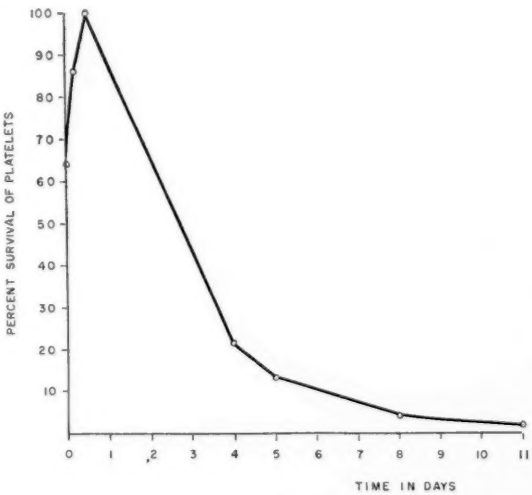

Fig. 5.—The survival of transfused radioactive platelets in recipient Dog 2.

1 mc. of P^{32} and 1 week later was phlebotomized. However, instead of carrying out the phlebotomy with plastic bags and EDTA as the anticoagulant, routine blood bank equipment was used (glass vacuum bottles with ACD solution). The blood was kept at 4° C. for 24 hours, then transfused. It is well known that such treatment results in nonviable platelets. When platelet activity in this recipient was measured, only the sample drawn 30 minutes after transfusion showed significant activity. Apparently some platelets survived for these few minutes, but by the fifth hour post transfusion these platelets had

disappeared. In Fig. 4, there is a comparison of platelet survival in this dog with platelet survival in Dog 1 shown in Fig 3.

Fig. 5 shows the survival of radioactive platelets in a second dog transfused with blood obtained by phlebotomy into a plastic bag. Platelet sequestration was even more marked and lasted longer in this animal than in Dog 1 (Fig. 3). In Dog 2, the peak in platelet activity did not occur until the end of day 1. The curve again appears to be exponential, this time with a 2½ day half-life and reaching the 10 per cent level on day 6. Some platelet activity was still present on day 11. It is unlikely that the radioactivity in platelets which persists so long after the transfusion could be due to adsorption of P^{32} from the plasma, because plasma activity in the recipient approaches background levels by the second or third posttransfusion day. An alternative explanation is that the residual activity is due to reutilization of phosphorus from platelets, red cells, and plasma. However, this source of phosphorus is so small in comparison with the total body phosphorus pool that the explanation seems unlikely. Furthermore, if reutilization were a significant problem, it would have evidenced itself in the dog that received nonviable platelets (Fig. 4). We believe this residual activity is because of a few transfused platelets that actually survive a long time. Perhaps these are the very youngest platelets in the transfused blood.

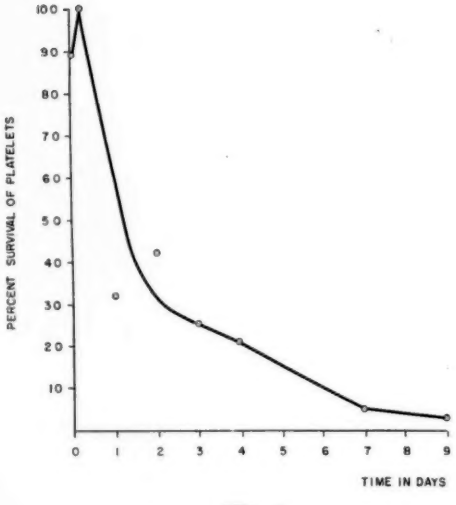

Fig. 6.

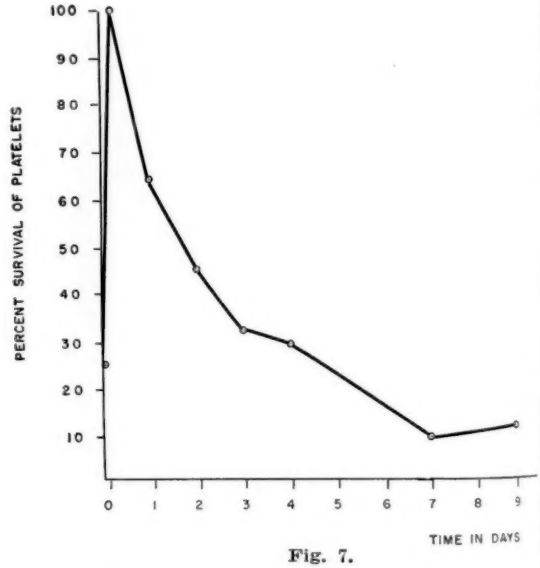

Fig. 7.

Fig. 6.—The survival of transfused radioactive platelets in recipient Dog 3.
Fig. 7.—The survival of transfused radioactive platelets in a normal human recipient (Patient 1).

The survival of radioactive platelets in a third dog is shown in Fig. 6. Here again there is evidence of sequestration, followed by an apparently exponential curve of decay. The half-life was short, 1½ days, and the 10 per cent level was reached on day 6.

Fig. 7 shows the typical survival of radioactive platelets in a normal *human* recipient. The amount of platelet sequestration that occurred after

the transfusion is striking. The curve of decay in the human subject also appears to be exponential. The half-life was 2 days, and the 10 per cent level was reached on day 7.

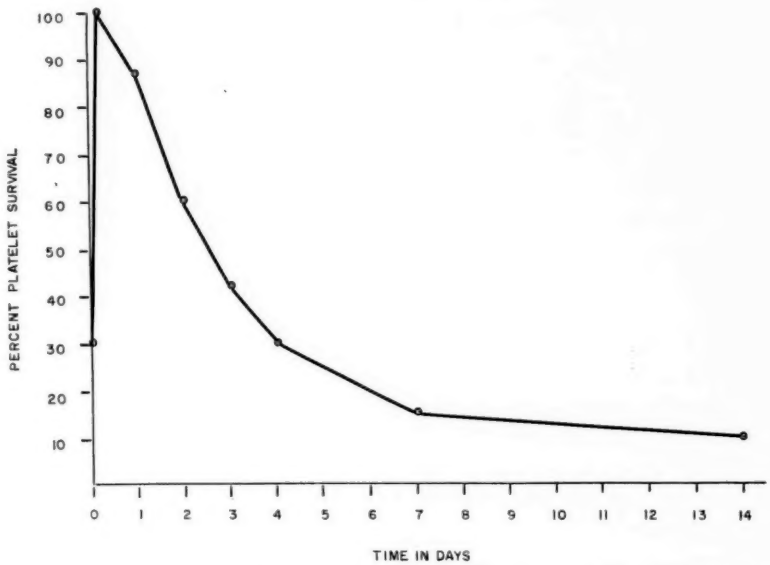

Fig. 8.—The survival of transfused radioactive platelets in a normal human recipient (Patient 2).

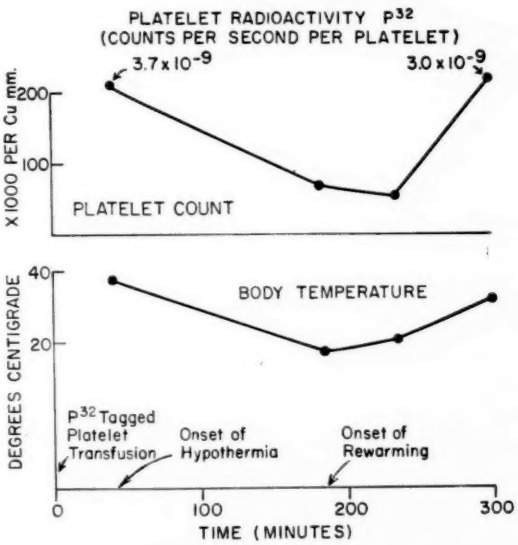

Fig. 9.—The sequestration of platelets during artificial hypothermia. Radioactive platelets were transfused into the recipient dog before his temperature was lowered.

Fig. 8 shows a second example of platelet survival in a normal human. Again there is an exponential curve. The half-life was 2½ days, and the 10 per cent level is reached on day 14. The sequestration was even more marked than in the dogs. We belive this to be the first time that platelet sequestration has been demonstrated.

Using the technique of tagging platelets with P^{32}, we have demonstrated a second example of platelet sequestration. In dogs undergoing deep hypothermia, we found nearly complete disappearance of platelets as the animals were cooled to 20° C., and a reappearance of platelets when they were rewarmed.[12] We transfused radioactive platelets into a dog which was then exposed to hypothermia (Fig. 9). The animal's platelet count dropped nearly to zero. On rewarming, the platelet count returned to normal. The radioactivity in samples of platelets obtained before and after chilling was nearly the same. The platelets which appeared after hypothermic thrombocytopenia were the same platelets which were in the blood before the dog was chilled. During deep hypothermia, platelets are sequestered.

SUMMARY

A new method of tagging platelets in vivo with P^{32} is presented. It is accomplished by injecting P^{32} into a donor, waiting until his platelet population contains a significant concentration of intrinsically labeled platelets and then transfusing his whole blood into a compatible recipient. The method has the advantage of requiring only minimal manipulation of the platelets. The curve of decay representing platelet destruction appears to be exponential both in humans and in dogs. The half-life is 1½ to 3 days, and the 10 per cent level of survival is reached in 6 to 14 days. Both in humans and in dogs there is evidence of a transient sequestration of platelets in the early posttransfusion period. A second example of platelet sequestration has been demonstrated by applying this technique of platelet tagging to the study of thrombocytopenia in hypothermic dogs.

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THE PERSISTENCE IN THE BLOOD OF THE RADIOACTIVE LABEL OF ALBUMINS, GAMMA GLOBULINS, AND GLOBULINS OF INTERMEDIATE MOBILITY

VI. The Uptake Plots of S^{35} Into Serum Protein Fractions During the First 24 Hours After Oral Administration of Labeled Methionine and Cysteine

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Chicago, Ill.

WHEN our studies[1, 2] of die-away plots (representing the metabolic degradation of S^{35} autosynthesized protein fractions) did not permit sure metabolic or clinical interpretation, our attention was directed to uptake plots (representing the incorporation of S^{35} into plasma protein fractions during the first 24 hours after oral administration) as a possible means of estimating synthesis rates. While free from the unmeasurable complication of isotope reutilization encountered in die-away plots, and while less tedious in execution, uptake plots nevertheless have all the pitfalls of tolerance tests.

It is the purpose of this paper: (1) to report our preliminary findings on the uptake of S^{35} into certain serum protein fractions during the first 24 hours after oral administration of labeled methionine and cysteine; and (2) to evaluate these findings in the light of clinical variables other than synthesis rates which may determine the form of an uptake plot, in order to see whether or not such variables (which are often hard to control) are sufficiently important to warrant promise for such plots in the clinical investigation of plasma protein metabolism.

METHODS

Subjects.—The 12 subjects studied fall into 4 diagnostic categories (Table I). These categories were chosen to cut across the possible clinical correlations for an uptake test.

The subjects with proved pancreatic disease represent deficiencies in dietary protein hydrolysis of varying severity as indicated by a range of serum albumin levels from 0.7 Gm./100 c.c. (Patient T. N.) to 3.4 Gm./100 c.c. (Au. J.). The ability to absorb the I^{131} label from oral albumin was, therefore, also measured on each patient in this group, as specified below and reported in Table I.

The patients in the second diagnostic category of Table I represent small bowel malabsorption of etiology ranging from the known (postoperative short bowel syndrome with

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stricture) to the unknown (regional ileitis and idiopathic nontropical sprue). Here also a range in serum albumin levels (0.8 Gm./100 c.c., Patient W. S. to 2.7 Gm./100 c.c., Patient J. S.) indicates varying severity of this defect.

All patients in the first two diagnostic categories exhibited steatorrhea.

The third category, which bears in Table I the heading "hypo- or dysproteinemias," includes the following cases.

1. Patient T. C. was deficient chiefly in the albumin fraction. This invited comparison with the findings of Kinsell and co-workers[3] in their patient with diagnosis idiopathic "hypoproteinemia." A previously published[4] die-away plot following I.V. albumin I^{131} for this patient yielded a shortened apparent turnover time for albumins comparable to the figure recently published for this diagnosis by Schwartz and Thomsen in Denmark,[5] as well as a depressed total exchangeable albumin pool.*

2. Patient A. R. exhibited deficiency chiefly in the gamma globulin fraction.† His idiopathically low level did not, however, approach the complete agammaglobulinemia of the patient we have previously reported in whom we could demonstrate no S^{35} uptake whatsoever in the gamma position.[6]

TABLE I*

DIAGNOSTIC GROUPS	PATIENT	MAXIMUM ALBUMIN I^{131} UPTAKE (% DOSE)	MAXIMUM S^{35} UPTAKE†			
			TOTAL PROTEIN	ALBUMINS	INTERMEDIATE GLOBULINS	GAMMA GLOBULINS
Pancreatic disease	T. N.	1.5	9,600	2,300	5,800	1,400
	Al. J	4.0‡	3,200	1.310	1,640	720
	Au. J.	4.0	3,500	1,350	2,000	500
	M. L.	6.5	3,400	1,200	1,800	550
	V. H.	4.5	3,600	1,280	1,750	480
Small intestinal malabsorption	L. D.	18.0	5,500	1,400	2,600	1,120
	W. S. (1)§	--	5,600	900	2,600	980
	(2)	9.0	5,100	1,800	3,400	600
	J. S.	12.0	3,500	1,050	2,750	400
Hypo- or dysproteinemias	T. C. (albumins)‖	11.2	9,200	6,500	3,300	1,100
	A. R. (γ globulins)	--	3,260	1,048	1,928	584
	J. F. (γ globulins) ¶	--	1,968	1,156	1,090	270
Nutritionally normal	J. M.§	15.1	5,950	2,600	2,200	750

*The diagnoses and paper electrophoretic albumin and globulin levels for 8 of the patients in Table I are given in Table I of the preceding paper in this series.[1] (Also, in Fig. 1 of this reference, die-away plots are presented for albumins and gamma globulins of 5 of these patients.)

In addition, Patient V. H. presented chronic calcific pancreatitis (autopsy). His serum protein levels were: total protein 6.0, albumin 2.8, and globulin 3.2 Gm./100 c.c. Patient J. S. presented regional ileitis (surgery). His serum protein levels were: total protein 6.0, albumin 2.7, and globulin, 3.3 Gm./100 c.c.

†This uptake (corrected counts per minute) is corrected to a standard 0.5 mc. oral dosage and is proportional to the true specific radioactivity in terms of mc./Gm.

‡Postpancreatectomy study. Preoperative albumin I^{131} uptake was 15.0 per cent.

§At initial labeling,[1] electrophoretic partition showed total protein 3.0, albumin 0.8, and gamma globulins 1.2 Gm./100 c.c. During remission, repeat labeling,[2] showed total protein 5.5, albumin 2.2, and gamma globulin 1.2 Gm./100 c.c.

‖Labeled with S^{35} cysteine 0.5 mc. All others received S^{35} methionine 0.5 mc.

¶This patient with multiple myeloma had, in addition, an "M" protein of 5.3 Gm./100 c.c., with a maximum S^{35} uptake of 342 c.p.m.

3. In Patient J. F. the deficiency was also chiefly in the gamma fraction, but was accompanied by a large fraction of slightly faster mobility characteristic of multiple myeloma.

*Reference 4 discusses the role of noncooperation with diabetic management in the etiology of this patient's hypoproteinemia. However, continuous ward observation for a period of nearly two years, with intensive efforts at supplementation, has led us to the belief that there is a further "idiopathic" factor.

†Judged by absence of isohemagglutinins and by history of multiple bacterial infections, this patient approached the state of "immunologic paralysis" although he demonstrated cutaneous sensitivity to tuberculin.

Finally there is included one metabolically normal subject (J. M.) whose die-away plots followed both the oral S^{35} and the intravenous I^{131} fractions we have already published.[1, 7]

Administration of Labels and Supplements; Radioactivity Measurements.—Two subjects (T. C. and J. M.) received oral S^{35} cysteine, 0.50 mc. All others received oral S^{35} methionine, 0.50 mc. Supplemental protein hydrolysates were given orally, in 2.5 Gm. doses at the time of labeling. Prolonged supplementation was not given. Sera were drawn at 1-, 2-, 4-, 8-, and 24-hour intervals. Methods of fractionation, extraction, measurement of component concentrations and radioactivity have been described previously. No significant differences were observed between radioactivity measurements on specimens before and after dialysis.[6]

Radioactivity uptakes were plotted on regular graph paper as corrected counts per minute against time in hours. By the corrections used these values are proportional to the specific radioactivities of the fractions in question (in terms of counts per minute per gram protein) and are corrected to a standard oral radioactivity dosage of 0.50 mc.

A maximum of 26,000 counts per minute in the "total protein" column of Table I would be achieved if 100 per cent of the oral dosage were present in a plasma volume of 2,500 c.c. at the time of the uptake plot maximum. Individual plasma volume measurements were not carried out in this study.

The albumin I^{131} absorbtion test was performed as described by Freeark, Kozoll, and Meyer.[8] Normal maximal absorption with this procedure is 14 per cent (range, 10 to 22 per cent) of the given dose.

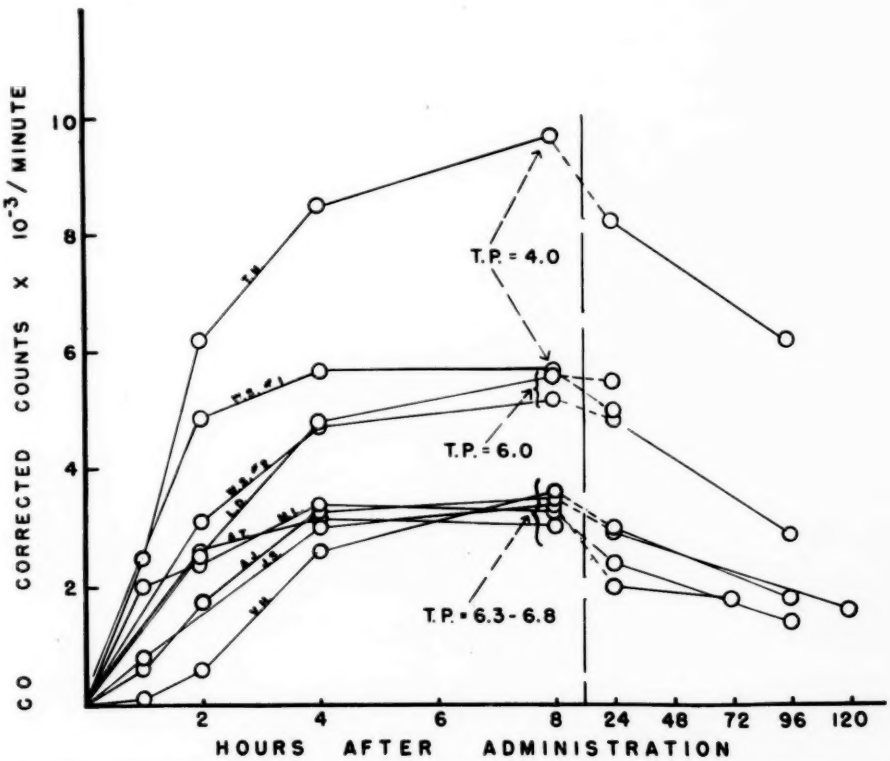

Fig. 1.—The incorporation of S^{35} methionine (500 μc) into unfractionated serum proteins during the first 24 hours after oral administration. The initials of specific patients are indicated on individual plots between hours 2 and 4, the total protein ranges between hours 6 and 8 above the abscissa.

RESULTS

Uptake of S^{35} by Unfractionated Serum Proteins.—The incorporation of S^{35} into the total serum protein reaches a maximum eight hours after oral administration. Fig. 1 illustrates this by the complete plots for 9 of the 13 studies whose maxima are given in Table I. Not infrequently a maximum level is reached at 4 hours. Kinsell[3] obtained similar results with intravenous S^{35} methionine.

Inspection of the left side of Fig. 1 indicates a lack of correlation between the initial steepness of plot and the type of digestive disturbance. The plots of greatest (T. N.) and least (V. H.) steepness both correspond to a common pathogenesis, pancreatic disease. The next steepest (W. S., 1) and the next flattest (J. S.) correspond to syndromes in which local absorptive problems might be expected to influence a tolerance test.

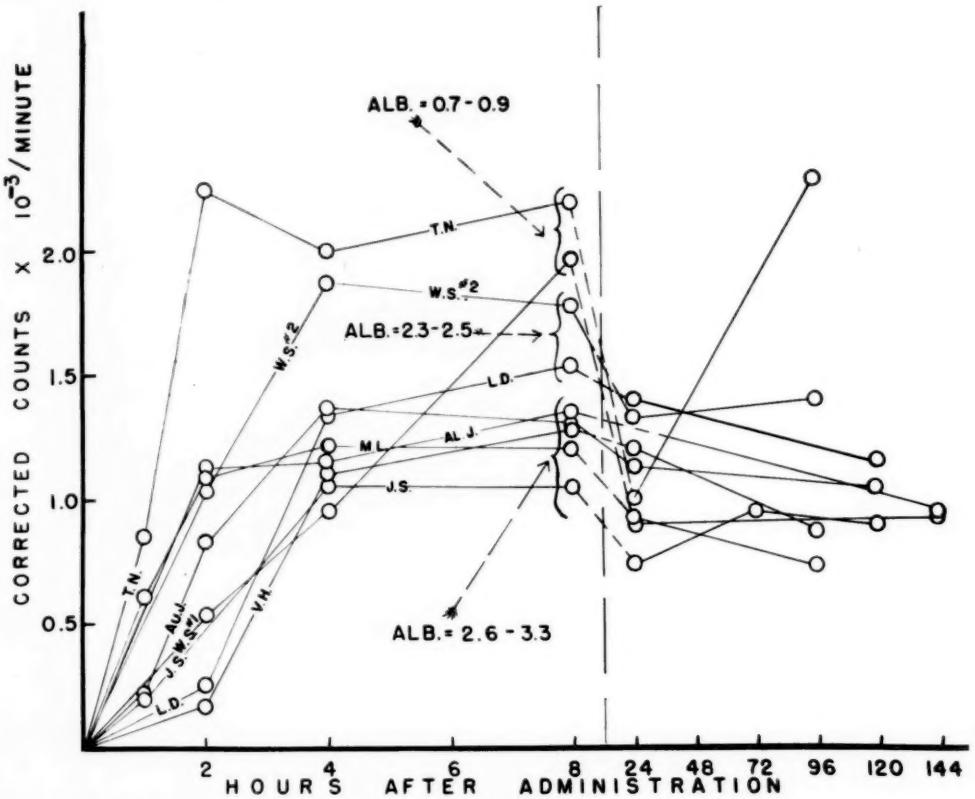

Fig. 2.—The incorporation of S^{35} methionine (500 μc) into the serum albumins during the first 24 hours after oral administration. Patients and serum albumin concentrations ranges are indicated essentially as in Fig. 1.

Inspection of Fig. 1 and Table I, shows that the maxima of the uptake plots may be inversely correlated with the level of serum total proteins. The highest maximum occurred in T. N., whose total protein was 4.0 Gm./100 c.c. In patient T. C. (not shown in Fig. 1), with a total protein of 3.7 Gm./100 c.c.,

a comparably high maximum is seen. L. D. shows a similar though somewhat lower maximum. Kinsell's demonstration[3] of an elevated uptake of S^{35} into the total plasma protein of a patient with idiopathic hypoproteinemia is thus extended to hypoproteinemia of another etiology.

However, this correlation does not always hold even in this small series. Patient W. S. was studied twice. In the first study, his total protein was 3.0 Gm./100 c.c., while in the second study, during a period of remission, his total protein had risen to 5.5 Gm. per cent after intensive nutritional therapy on the ward. The 8 hour uptake maximum, however, was alike in both studies.

Uptake of S^{35} into Serum Albumins.—The highest maxima occurred in uptake plots of patients with the greatest depressions of the serum albumin level (e.g., Patient T. N. in Fig. 2 with an albumin level of 0.7 Gm./100 c.c.) In Patient T. C. (not illustrated), with an albumin level of 1.3 Gm./100 c.c., an uptake three times as great as this was achieved after oral S^{35} cysteine. Again, Patient W. S., in duplicate studies, presented similar uptakes, although his albumin level had risen from 0.9 to 2.3 Gm./100 c.c. For patients with lesser degree of hypoalbuminemia, the corresponding differences in degree of uptake were less significant. As with the total serum proteins, there thus appears a rough inverse proportionality of uptake maximum to the level of serum albumin. (Fig. 2 presents complete plots for 9 of the studies whose maxima are given in Table I.)

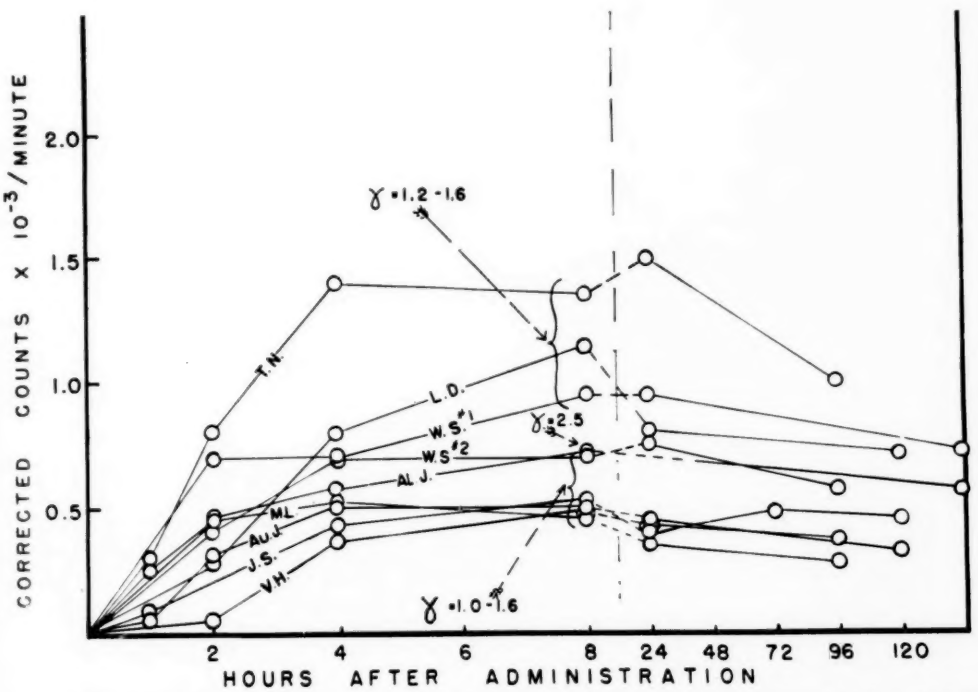

Fig. 3.—The incorporation of S^{35} methionine (500 μc) into the gamma globulins during the first 24 hours after oral administration. Note, in contrast to Figs. 1 and 2, that the order of maxima of the plots shows no correlation with order of gamma globulin concentrations.

Uptake of S^{35} into Gamma Globulins.—While in general uptake maxima were significantly lower than for corresponding albumins (compare Figs. 2 and 3), in contrast to the albumins, no relationship could be observed between these maxima and the levels of serum gamma globulin (Fig. 3 and Table I).

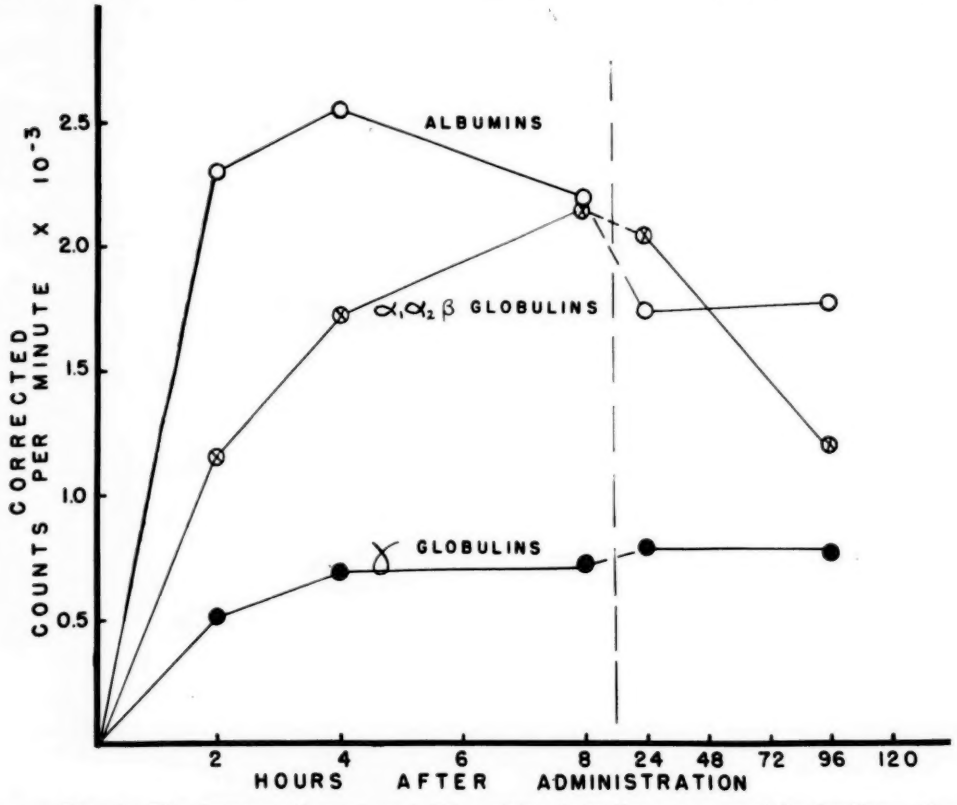

Fig. 4.—The incorporation of oral S^{35} cysteine into the serum protein fractions of a nutritionally normal patient (J. M.). Note that the gamma globulin maximum is much lower, and the alpha-beta maximum about the same as the albumin maximum.

The highest maximum occurred in nutritionally normal Patient J. M. (Fig. 4, note S^{35}-cysteine label). However, both T. C. and T. N. (who showed highest total protein and albumin maxima) also presented high gamma globulin maxima when compared to the others in the group.

Uptake of S^{35} into Globulins of Intermediate Mobility.—The plot maxima for this crude mixture of alpha-1, alpha-2, and beta globulins (Table I) have been in general greater than the corresponding albumins maxima save in the two instances where S^{35} cysteine was used as the oral label. Fig. 4 presents, for Patient J. M., uptake plots which illustrate this point in comparison with those in Fig. 5 (Patient J. S.).

While these high maxima are of interest in the light of the short apparent turnover times we have already published for this fraction, because of its crudity we will limit our discussion at this time to the albumins and gamma globulins.

I^{131} Uptake from Oral Albumin I^{131}.—The results of this procedure are given in Table I. All the patients with chronic pancreatic disease showed difficulty in absorbing an albumin I^{131} test meal. Patient L. D., with idiopathic sprue, is of especial interest since she not only did not show any difficulty in absorbing the label from oral albumin I^{131}, but also little difficulty in S^{35} amino acid uptake. The same is true for Patient J. S. (regional ileitis).

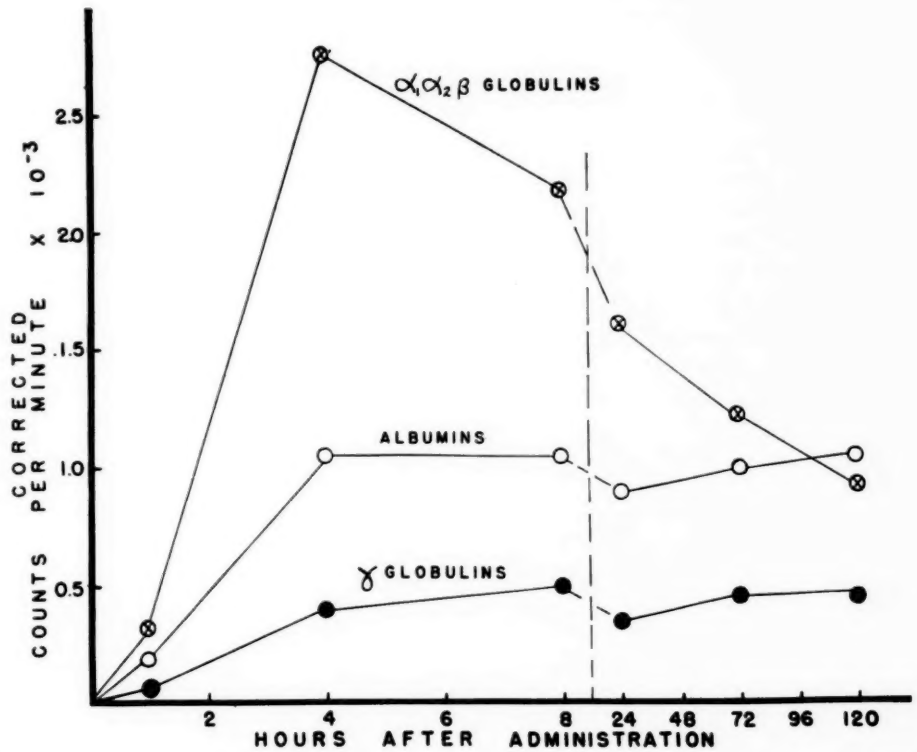

Fig. 5.—Incorporation of oral S^{35} methionine into the serum protein fractions of a patient (J. S.) with regional ileitis and steatorrhea. Note that while gamma and alpha-beta maxima are similar to Fig. 4, the albumin maximum represents less than one-half the Fig. 4 value.

DISCUSSION

Given a standard dosage, the form of an oral tolerance plot is clearly a function of rates of absorption as influenced by both gastrointestinal motility and local conditions. Our oral uptake plots for total proteins resemble those published by Kinsell and co-workers following intravenous administration of S^{35} methionine. The resemblance suggests such rapid intestinal absorption that oral labeled amino acids probably do not hold as promising a future role in clinical elucidation of absorption defects as do the I^{131}-labeled lipids.[9-11]

Further groups of variables which influence the form of the plot include: (a) rates of equilibration with extravascular body compartment; (b) rates of excretion by the kidney; and (c) rates of metabolic turnover. Such a range

of uncontrolled variation makes it amazing that tolerance tests have been capable of any useful clinical correlation. When an isotopic label is used a further variable is emphasized: namely, the circulating pool size of the unlabeled component to which the newly introduced label is added.

Thus given hypothetically identical dosages, absorptions, and rates of incorporation into serum albumin, if the liver delivers the same amount of S^{35} albumin into identical plasma volumes of 2 subjects, one with an albumin level of 3 Gm./100 ml., the other with a level of 1 Gm./100 ml., the specific radioactivities, and thus probably the plot maxima, will be one third as high for the first subject as for the second. This problem in more complex form is clearly recognized by Gould and co-workers[12] as a limitation in clinical estimation of the rate of cholesterol synthesis from uptake plot maxima following C^{14} administration. These authors proposed correction procedures which might give their tolerance test "value as a clinical laboratory test." Kinsell used such a correction in interpreting the twice normal S^{35} uptake maxima in his patient with idiopathic hypoproteinemia and concluded that the defect is "hypercatabolism not compensated by hyperanabolism." If we apply correction factors based on individual serum electrophoretic levels to the maxima of Table I, the resultant maxima should be, to a first approximation, independent of pool size, and thus a rough measure of the synthesis rate of the component in question.

TABLE II

PATIENT	LEVELS (GM./100 C.C.) ALBUMINS	GLOBULINS	OTHER	CORRECTED RADIOACTIVITY MAXIMA ALBUMINS	GLOBULINS	OTHER	SUGGESTED INTERPRETATION
				Hypo-albuminemias			
T. N.	0.7	1.6	--	420	1,720	--	Depressed albumins synthesis and accelerated gamma globulin synthesis related to pancreatic disease and hepatic disease
W. S.(1)	0.8	0.9	--	190	900	--	Depressed albumin synthesis related to malnutrition of chronic small bowel disease, restored toward normal by dietary therapy
W. S.(2)	2.2	0.9	--	1,030	550	--	
T. C.	1.3	0.7	--	2,200	810	--	Accelerated albumin synthesis in the face of chronic hypoproteinemia resistant to dietary therapy, pointing to a hypercatabolic albumin utilization without comparable gamma globulin abnormality
				Hypogammaglobulinemias			
A. R.	4.2	0.3*	--	1,140	140	--	Depressed gamma globulins synthesis in the face of normal albumin synthesis
J. F.	2.1	0.55	5.3†	632	115	1,395†	Depressed gamma globulins synthesis in the face of an elevated synthesis of the abnormal "M" protein; probable depression of albumin synthesis

*This value was obtained at time of uptake study. Subsequent serial observations have yielded a range between 0.1 and 0.4 Gm./100 c.c.

†"M" protein, intermediate in mobility between gamma and beta globulins. The correction factor used was based on the normal circulating gamma globulins pool. If the circulating albumins pool is used for the correction factor, the correct maximum becomes 470 counts per minute.

The roughness of the first approximation is indicated by the fact that the correction factors neglect unmeasured variability in plasma volume and in renal and fecal losses of the labeled amino acid as well as losses of the labeled protein component to extravascular compartments.*

Table II (left side) presents the results of such correction to the albumin and gamma uptake maxima for the 5 patients of Table I for which correction factors are greatest.† These 5 patients include 3 hypoalbuminemias and 2 hypogammaglobulinemias.

It will be seen, for the former, that a deficient level has been found associated with uptake maxima both exceedingly low and quite high. Suggested interpretations (Table II, right side) indicate our belief in the possibility of thus differentiating a hypercatabolic from a deficient level due to other causes.

Maxima for the hypogammaglobulinemias have been exceedingly low. It is tempting to interpret the low gamma globulin uptake in patient J. F. (myeloma) as an incapacity of the plasma cell mass to put out normal gamma globulins in the face of synthesis of "M" protein. In favor of this is the high "M" protein uptake and the normal albumin uptake (Table II, bottom line).

We recognize that the maxima of these uptake plots may not reflect capacity of a given organ system for synthesis of a given protein component. For avidity in another organ system, rather that incapacity in the one, may so influence the distribution of labeled amino acid as to make estimates of defects in synthesis capacity undetectable.

SUMMARY

Because of our failure to achieve reliable metabolic and clinical interpretations of the die-away plots of autosynthesized plasma protein fractions following oral S^{35} amino acid administration, our attention has turned to the uptake plots of radioactivity during the first 24 hours after feeding the label. Such plots are subject to the multiple variables and interpretive pitfalls of tolerance tests, and are discussed in these terms.

Plots are reported for a small pilot group of twelve subjects divided between four diagnostic categories. The subjects were chosen: (a) to represent samples of the clinical spectra of hypoalbuminemia and hypogammaglobulinemia; (b) to include a range of hypoalbuminemias on the basis of chronic pancreatic deficiency, as well as the "idiopathic" and small intestinal malabsorption type; and (c) to include subjects with normal serum concentrations of gamma globulins in the presence of albumin deficiency, and vice versa.

No significant dialyzable radioactivity appeared in the blood stream after the oral S^{35} amino acids. This suggests very rapid incorporation of absorbed dosage into serum proteins and/or segregation intracellularly. Nondialyzable serum radioactivity appeared within an hour.

*The latter losses are probably sufficiently small as compared to the differences in uptake maxima, as to warrant neglect of the complex mathematical treatment[13] at this stage of our work.

†The correction factors, by which the Table I maxima are multiplied, represent the ratios of the observed electrophoretic concentration to the normal serum concentration of that component.

Based on standardized label dosage, specific radioactivity maxima were achieved in about eight hours and were, in all instances, higher for albumins than for corresponding serum gamma globulins.

While it proved possible to distinguish pancreatic hypoalbuminemias from both small intestinal malabsorption and from a normal subject on the basis of absorbed radioactivity from the oral intact protein albumin I^{131}, no such differentiation could be accomplished by the initial slopes (representing rapidity of absorption) of the S^{35} amino acid uptake plots.

When uncorrected for the circulating pool of unlabeled protein component to which the newly synthesized S^{35} protein is added, albumins uptake maxima, in general, appeared inversely proportional to the degree of hypoalbuminemia.

While reflected in the uptake maxima for unfractionated serum proteins, such an inverse relationship did not appear applicable for gamma globulins.

When maxima for the albumins and gamma globulins were subjected to an arbitrarily estimated correction for unlabeled circulating pools (a) the maxima for hypogammaglobulinemias were strikingly depressed as compared to the maxima for those subjects with a normal serum concentration of this electrophoretic component, while; (b) the instances of hypoalbuminemia included maxima both strikingly lower and considerably higher than those for subjects with more normal serum albumin concentrations.

It is suggested that these maxima may bear a rough relation to the rates of synthesis of the component in question, and that the spread of albumins maxima suggests the possibility of clinically distinguishing "hypercatabolism" from other mechanisms.

While it is recognized that uptake plot maxima can never be a measure of maximum synthesizing *capacity* of an organ system, and merely represent a very rough approximation to the resultant of many simultaneous processes, nevertheless, these findings seem sufficiently encouraging to warrant further clinical exploration in a larger series of defects in the metabolism of plasma proteins.

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THE EFFECT OF L-NOREPINEPHRINE ON URINE COMPOSITION AND ACID-BASE EQUILIBRIUM IN MAN

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THE adrenal medullary hormones, l-epinephrine and l-norepinephrine, elicit acute alterations in renal function when administered in physiologic dosage to normal man. Both induce intrarenal vasoconstriction and a reduction in renal blood flow, a sustained or increased urine volume, and an augmented tubular reabsorption of sodium and potassium.[1-3] The effect on urine flow has been attributed, at least in part, to suppression of the secretion of the antidiuretic hormone of the posterior pituitary.[4, 5] The factors contributing to the accelerated tubular electrolyte transport are not clear. This action of the medullary hormones appears to be independent of their pressor activity and also of secondary adrenal cortical secretion and renal neurogenic activity.[4] The electrolyte retaining effect of these substances has not, however, been demonstrated independently of the accompanying intrarenal circulatory adjustments[4] nor has the change in tubular activity been separated from the systemic metabolic alterations elicited by these hormones.

These alterations in sodium and potassium output have been reported to occur in association with other changes in urine composition. Chloride excretion has been found to diminish[3] and urinary pH to increase.[6] However, a systematic study of the effects of the medullary hormones on urinary acid-base excretion has not been made. Alterations in plasma acid-base balance, consisting of an early, transient respiratory alkalosis[7, 8] and a later and more prolonged metabolic acidosis,[8-10] are elicited by these agents, but the relationship between these adjustments and the changes in urine composition is not known.

These considerations lead to the present investigation which was undertaken in order to determine the effects of l-norepinephrine on urinary acid-base excretion and to study the relationship between the changes in urine and plasma composition.

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METHOD

The effects of l-norepinephrine on urinary and plasma acid-base composition were determined in 12 nomal human subjects. The effects of ephedrine sulfate were observed in three additional subjects. The subjects varied in age from 21 to 58 years and were free from apparent cardiovascular or renal disease. Each was maintained on a regular hospital diet containing approximately 10 Gm. sodium chloride daily.

All studies were made in the morning after an overnight fast and after the subject had ingested 500 ml. of water. Urine was collected under mineral oil through a multi-eyed urethral catheter. At the mid-point of each period enough urine was collected for the determination of pH and CO_2 content. At the end of each period the bladder was irrigated with distilled water and emptied as completely as possible by manual compression. Blood was withdrawn anaerobically at appropriate intervals from a brachial artery (10 subjects) into oiled heparinized syringes. In 5 subjects (D. H., E. P., E. C., J. S., and M. G.) samples of blood were taken from an antecubital vein following immersion of the forearm in water at 47° C. for 10 minutes. Blood was centrifuged immediately and the plasma was stored in a refrigerator until analysis could be made, which was usually within 4 hours.

Glomerular filtration rate was measured as the inulin clearance.[11] The pH of plasma and urine were determined anaerobically at room temperature with a Cambridge glass electrode pH meter. Plasma pH was corrected to body temperature using a factor of 0.01 pH unit per degree centrigrade.[12] Urine pH was not corrected to body temperature.[12] Urine and plasma were analyzed for total CO_2 content by the method of Van Slyke and Neill.[13] Bicarbonate and carbonic acid concentrations were calculated from the Henderson-Hasselbach equation using a value of 6.1 for pKa′. Carbon dioxide tension was calculated from the carbonic acid content, using a value of 0.301 and 0.0309 for α in plasma and urine, respectively.[14] Urinary titratable acid was determined with an external glass electrode, titrating to the pH of plasma with 0.01 N NaOH. The ammonia content of urine was determined by a modification of the method of Van Slyke and Cullen.[15] The urine of two subjects (S. G. and E. T.) was analyzed for organic acids by the method of Van Slyke and Palmer.[16] However, a correction for creatinine was not made. Urine and plasma were analyzed for chloride by the method of Schales and Schales[17]; for sodium and potassium in an internal standard flame photometer; and for inorganic phosphate by the method of Fiske and Subbarow.[18]

Following the determination of control values during 2 or 3 successive 10- to 20-minute intervals, l-norepinephrine (8 μg per ml. 5 per cent dextrose in water) or ephedrine sulfate (2 μg per ml.) was administered intravenously at a rate sufficient to maintain systolic blood pressure, determined sphygmomanometrically, at least 25 to 50 mm. Hg above control values. The amount of drug employed varied from 1 to 30 μg per minute of l-norepinephrine and from 0.5 to 6.0 mg. per minute of ephedrine. Blood pressure was allowed to stabilize from 10 to 20 minutes and urine collected during this interval was discarded. Two or three clearance periods, 10 to 20 minutes in length, were then obtained during infusion of the pressor agent. One or two "recovery" periods were obtained after the drug was discontinued in all subjects but D. C. and D. T. Blood pressure and pulse rate were recorded every one to two minutes during and following infusion of the pressor agent.

The response to l-norepinephrine was examined in 6 subjects who were in normal acid-base balance and in 3 who had been given 6 to 12 Gm. of ammonium chloride orally over an 8- to 12-hour period on the day prior to the study. Ammonium chloride was not given within a minimum of 4 hours, and usually not within 8 hours of the beginning of the experiment.

Three additional subjects received an infusion of 2.25 per cent sodium bicarbonate at a rate (4 ml. per minute) sufficient to increase progressively the CO_2 content of plasma throughout the study. Two of these subjects (F. G. and E. P.) had received ammonium chloride the day previously. The response to ephedrine was studied in three subjects, two of whom (E. C. and J. S.) had received ammonium chloride.

RESULTS

Response to l-Norepinephrine.—The changes in acid-base composition of urine and plasma induced by l-norepinephrine are presented in Table I. Plasma

TABLE I. THE EFFECT OF L-NOREPINEPHRINE ON

SUBJECT SEX	S.A. AGE	PROCEDURE	B.P. (MM./HG)	P.R.	PLASMA Na (MEQ./L.)	K (MEQ./L.)	Cl (MEQ./L.)	PO_4 (MM./L.)	HCO_3 (MM./L.)	pCO_2 (MM./HG)	pH (UNITS)
B.W.		Control	120/70	52	135	3.8	108	0.89	25.6	39.5	7.44
M 36	1.96	Infusion	159/81	50	135	3.6	106	0.72	24.2	37.5	7.44
		Recovery*	124/70	70	137	--	108	0.69	24.2	36.2	7.45
D.H.		Control	98/58	80	136	3.7	109	0.77	24.8	43.2	7.38
F 26	1.74	Infusion	144/88	54	129	3.8	106	0.72	23.8	41.5	7.39
		Recovery	96/50	66	129	3.5	105	0.75	24.3	41.9	7.38
M.G.		Control	110/60	76	134	3.6	110	--	25.5	35.9	7.49
F 21	1.60	Infusion	146/88	60	136	3.5	106	--	23.8	33.2	7.49
		Recovery	106/68	72	135	3.5	110	--	25.4	34.2	7.48
S.G.		Control	109/70	77	137	4.8	109	1.00	22.8	35.6	7.43
M 40	1.62	Infusion	170/100	60	134	4.7	107	0.80	21.5	32.2	7.44
		Recovery	110/68	71	134	4.6	103	0.80	22.4	33.6	7.44
O.G.		Control	98/68	72	142	4.1	108	1.16	26.3	39.9	7.44
M 31	1.68	Infusion	136/80	64	141	3.9	106	1.09	25.6	39.2	7.43
		Recovery*	90/60	72	139	4.1	106	1.07	26.1	39.5	7.43
D.C.		Control	100/70	80	131	4.2	104	1.07	25.2	41.9	7.44
M 49	1.68	Infusion	141/92	72	133	4.2	106	1.11	24.2	39.9	7.41
A.C.		Control	107/68	54	132	4.2	105	0.90	25.6	45.2	7.37
M 37	1.88	Infusion	168/90	48	132	4.2	107	1.00	25.1	44.2	7.37
		Recovery	112/70	54	134	4.0	105	1.00	25.0	43.5	7.37
E.T.		Control	120/72	90	133	4.3	105	0.83	21.5	38.2	7.35
M 37	1.79	Infusion	172/90	66	133	4.2	105	0.87	19.3	41.5	7.28
		Recovery	120/70	82	133	4.1	109	0.85	21.0	38.2	7.36
A.M.		Control	120/78	96	131	3.9	112	0.87	17.9	34.2	7.34
F 42	1.66	Infusion	164/90	78	129	4.1	110	0.93	17.1	32.2	7.32
		Recovery	106/76	118	131	4.1	112	0.83	17.9	34.6	7.32

Key to abbreviations: S.A. = body surface area (M^2); P.R. = pulse rate; V = urine flow (ml./min); GFR = glomerular filtration rate, inulin clearance (ml./min.); T.A. = titratable acid (mEq./min.); A. C., E. T., and A. M. had received ammonium chloride on the day prior to the study.

*All values are averages of two or more determinations except in instances marked with an asterisk. L-norepinephrine was administered during the "infusion" period at a rate sufficient to produce the desired elevation of arterial pressure, which was measured sphygomanometrically. The values of various urinary constituents represent excretory rates except for pH and pCO_2.

bicarbonate content decreased slightly, carbon dioxide tension fell, and the acidity of plasma was unchanged. The reduction in bicarbonate concentration varied from 0.7 to 1.7 meq. per liter, averaged 1.1 meq. per liter, and was highly significant ($p < 0.01$). During recovery all values returned promptly toward control levels. The plasma concentrations of sodium, potassium, chloride, and phosphate did not change.

The urinary excretion of sodium and potassium diminished during l-norepinephrine infusion in 4 of 6 subjects. This reduction in cation excretion was accompanied largely by a diminished excretion of chloride, and in D. H. and M. G. by a reduction in bicarbonate output as well. Titratable acid, inorganic phosphate, and ammonium excretion and urinary pH showed no consistent change. In some instances (D. H., S. G., A. C., and D. C.) bicarbonate output appeared to vary with changes in urine flow rather than with changes in sodium and potassium output. This correlation has been noted by others.[12, 19] Organic acid excretion, not presented in the table, did not change in the two subjects (S. G. and

ACID-BASE EQUILIBRIUM AND URINE COMPOSITION*

GFR (ML./MIN.)	URINE V (ML./MIN.)	pH (units)	Na (μEq/MIN.)	K (μEq/MIN.)	cl (μEq/MIN.)	PO_4 (μM/MIN.)	HCO_3 (μM/MIN.)	pCO_2 (mM./Hg)	T.A. (μEq/MIN.)	NH_4 (μEq/MIN.)
114	10.4	5.61	101.5	43.3	0	8.3	5.6	56.0	25.3	38.9
114	10.2	5.08	86.8	37.4	0	6.1	1.7	55.5	33.9	41.3
101	7.7	5.29	95.8	24.3	0	6.0	2.0	51.5	22.5	32.1
123	9.7	6.72	137.7	72.7	77.9	6.9	45.1	35.8	5.0	14.8
95	5.3	6.60	90.3	30.2	64.0	6.1	18.5	35.8	6.5	24.3
112	3.0	6.23	52.9	17.7	41.9	7.8	6.4	35.2	8.4	18.3
153	6.5	6.59	271.8	70.3	200.0	--	21.9	34.2	8.9	32.9
116	6.6	5.82	206.0	55.4	103.9	--	4.1	38.2	14.9	34.7
127	7.4	5.68	245.8	60.0	141.5	--	3.4	36.9	19.0	36.8
104	4.7	6.75	75.4	49.4	71.7	3.7	18.7	30.8	--	13.5
150	7.0	6.82	84.7	54.6	88.8	1.6	32.0	28.4	--	10.3
108	4.2	6.17	28.7	38.3	40.3	1.2	6.1	49.9	--	11.2
77	5.9	6.35	68.3	65.9	108.0	7.8	11.1	34.3	6.3	19.9
107	7.1	6.18	79.9	53.3	118.0	10.0	9.0	34.7	8.6	22.1
71	3.1	6.08	82.1	53.7	128.0	13.2	3.0	44.7	8.3	10.1
124	3.1	6.68	145.0	38.8	126.0	8.6	23.4	63.4	6.7	13.9
139	7.0	6.48	134.0	37.7	110.0	14.3	26.6	54.9	17.7	17.4
136	4.4	5.35	205.2	79.2	301.7	5.7	1.2	43.9	17.5	45.0
133	9.2	5.55	144.6	68.7	207.7	11.4	4.7	56.7	22.5	44.0
131	6.8	5.61	148.9	55.0	204.8	12.5	1.8	44.3	14.0	46.0
140	7.2	5.79	262.8	69.3	353.3	0	5.9	49.0	--	45.7
120	7.9	5.82	76.0	40.9	136.5	0	6.1	47.5	--	45.3
178	3.4	5.41	137.2	44.0	207.9	1.6	2.0	36.4	--	20.3
140	11.0	5.29	193.0	41.2	250.0	14.3	2.3	43.4	22.9	32.8
61	10.2	5.13	124.8	36.9	165.9	17.1	1.5	42.3	26.2	31.5
118	3.9	5.17	91.3	20.2	130.9	10.9	0.5	35.5	16.6	25.1

E. T.) in whom it was measured. Gross deficits of either cation or anion output were not encountered. The anion deficit apparent in subject B. W. was the result of the presence of Diodrast which was administered as part of another study. Subject M. G. also had received Diodrast.

In two subjects (S. G. and O. G.) urinary electrolyte excretion, which was low in the control periods, increased in association with a rise in glomerular filtration rate. This response was unlike that usually elicited by the adrenal medullary hormones. However, in these subjects electrolyte output increased less than filtration, suggesting an augmented tubular reabsorption per unit of filtrate. No consistent alteration in urinary acid-base equilibrium was noted in these subjects.

The withdrawal of l-norepinephrine was usually followed by a reduction in urine volume (Tables I and II), which, in most, but not all, instances had been sustained or had increased during infusion of the drug. Sodium, potassium, and chloride output tended to return toward control levels or continued to fall at this time. Bicarbonate excretion typically diminished, usually in association with the decline in urine flow, and the urine became more acid. Titratable acid, ammonium, and phosphate excretion did not change significantly.

TABLE II. THE EFFECT OF L-NOREPINEPHRINE ON ACID-BASE EQUILIBRIUM

SUBJECT SEX	S.A. AGE	PROCEDURE	B.P. (mM./ Hg)	P.R.	PLASMA Na (mEq./ L.)	K (mEq./ L.)	Cl (mEq./ L.)	PO_4 (mM./ L.)	HCO_3 (mM./ L.)	pCO_2 (mM./ Hg)	pH (units)	GFR (ML./ MIN.)
F.G.		Control	115/68	74	137	3.9	113	1.1	21.2	37.5	7.35	118
F	36 1.59	Infusion	154/82	72	137	3.6	109	0.8	23.3	39.9	7.37	104
		Recovery	130/70	--	137	3.1	111	0.8	24.2	43.9	7.41	116
E.P.		Control	100/62	60	133	3.9	110	1.2	24.6	43.5	7.39	141
F	37 1.62	Infusion	152/75	54	135	3.3	103	1.1	24.8	42.2	7.41	94
		Recovery	90/50	70	139	3.5	106	1.0	27.6	43.5	7.43	99
J.R.		Control	110/70	78	138	3.7	108	1.1	25.3	41.2	7.44	105
M	58 1.69	Infusion	152/80	72	--	3.6	108	1.2	25.6	44.2	7.40	107
		Recovery	110/60	--	141	--	110	0.5	26.7	36.9	7.47	100

See Table I for key to abbreviations.

*All values are avergaes of two or more determinations except for the recovery period. A continuous infusion of 2.25 per cent sodium bicarbonate was administered throughout the study. L-norepinephrine was administered during the "infusion" period. Subjects F. G. and E. P. had received 8 Gm. of ammonium chloride on the day prior to the study.

Ammonium chloride was administered in three subjects in order (1) to increase titratable acid and ammonium output to levels at which changes during l-norepinephrine administration would be more easily interpretable and (2) to assure an adequate excretion of cation. The dosages employed produced a slight reduction in the pH of plasma in two subjects (E. T. and A. M.) but were insufficient to produce excessive alteration in urinary composition (Table I). The changes induced by l-norepinephrine under these circumstances closely resembled those observed in subjects who did not receive ammonium chloride (Table I). Titratable acid and ammonium output were not affected significantly and sodium, potassium, and chloride output diminished abruptly.

In an attempt to separate the alterations in urine composition from the changes in plasma acid-base composition induced by l-norepinephrine an infusion of 2.25 per cent sodium bicarbonate was administered intravenously to three subjects at a rate sufficient to prevent the usual fall in plasma bicarbonate content elicited by l-norepinephrine. The results of this study are presented in Table II. Plasma bicarbonate concentration increased progressively throughout the study in each instance. Other plasma constituents were not affected significantly during the administration of sodium bicarbonate except for a tendency for the pH to increase and for sodium concentrations to rise and chloride concentrations to fall. The typical changes in urine composition elicited by l-norepinephrine occurred in these subjects. Sodium, potassium, and chloride output fell and no consistent change in urinary pH or ammonia or titratable acid excretion occurred.

Response to Ephedrine.—Ephedrine, which resembles the adrenal medullary hormones chemically but which differs in its effects on renal hemodynamics and electrolyte excretion,[4] was administered to 3 subjects (Table III) in order to evaluate further its effects on renal electrolyte metabolism.

The bicarbonate content of plasma and pCO_2 remained constant in two subjects (D. T. and E. C.) during the pressor response to ephedrine. In the third

AND URINE COMPOSITION DURING INFUSION OF 2.25 PER CENT SODIUM BICARBONATE*

	URINE									
GFR (ML./MIN.)	V (ML./MIN.)	pH (units)	Na (μEq/MIN.)	K (μEq/MIN.)	Cl (μEq/MIN.)	PO_4 (μM/MIN.)	HCO_3 (μM/MIN.)	pCO_2 (mM./Hg)	T.A. (μEq/MIN.)	NH_4 (μEq/MIN.)
118	3.5	5.20	193.0	113.5	325.3	9.4	0.7	35.8	16.5	--
104	5.1	6.28	21.6	47.1	37.8	4.1	7.8	32.4	6.8	--
116	1.5	5.88	78.5	36.7	64.7	11.4	1.1	40.1	6.5	--
141	6.4	5.13	192.8	109.0	313.5	17.2	1.1	42.3	24.4	45.4
94	3.5	6.59	39.5	52.2	63.5	10.2	11.7	33.0	7.5	16.7
99	0.7	6.48	53.4	39.6	47.0	14.4	2.5	47.6	5.4	8.9
105	5.8	6.96	74.8	110.9	108.9	11.4	53.4	43.4	6.1	6.8
107	6.3	6.66	35.6	75.6	45.7	9.9	33.1	46.4	12.2	7.1
100	4.9	6.58	27.6	55.7	34.5	8.1	19.7	41.8	10.6	7.5

subject (J. S.) the bicarbonate content of plasma decreased and fell further after the drug was discontinued. The sustained elevation of arterial pressure at this time in J. S. and in E. C. suggests continued pressor and perhaps metabolic activity of ephedrine during the "recovery" period. The acidity of plasma was unaltered during the infusion of ephedrine and the plasma concentrations of sodium, potassium, chloride, and inorganic phosphate remained constant. The changes in plasma composition thus resembled those elicited by the adrenal medullary hormones, but were less consistent, an observation which might have been predicted from previous observations of the effects of this agent on ventilation[20, 21] and on carbohydrate metabolism.[22] Urinary sodium and chloride output increased in two subjects (D. T. and E. C.) in association with an increment in glomerular filtration rate and diminished in the third study in association with a fall in filtration. Sodium output increased more than filtration in subjects D. T. and E. C., suggesting diminished tubular reabsorption per unit of filtrate. The sodium-inulin clearance ratio did not change in J. S. Potassium output fell progressively in all three studies. These results are in substantial agreement with previous observations[4] of the effects of ephedrine on electrolyte excretion, except that changes in filtration were more prominent in the present study. No consistent effect was noted on urinary acidity and CO_2 content or on titratable acid, ammonium or inorganic phosphate excretion.

DISCUSSION

L-epinephrine increases transiently the depth and rate of respiration when administered in physiologic amounts to man and induces a mild respiratory alkalosis.[7, 9] Later, lactic acid accumulates in extracellular fluid secondary to accelerated glycogenolysis[8] in muscle, and extracellular bicarbonate content remains reduced despite apparent correction of the respiratory alkalosis.* L-norepinephrine induces similar but somewhat less pronounced changes,[7, 26] although alterations in plasma lactic acid content are not a prominent feature

*The plasma concentration of other organic acids may also increase secondary to muscular activity during overventilation,[23, 24] However, in the present study urinary organic acid excretion did not change.

TABLE III. THE EFFECT OF EPHEDRINE SULFATE

SUBJECT SEX	AGE	S.A.	PROCEDURE	B.P. (mM./Hg)	P.R.	PLASMA Na (mEq./L.)	K (mEq./L.)	Cl (mEq./L.)	PO_4 (mM./L.)	HCO_3 (mM./L.)	pCO_2 (mM./Hg)	pH (units)
E.C.			Control	116/78	76	135	4.0	109	0.9	25.1	38.5	7.44
F	48	1.49	Infusion	169/90	74	135	3.4	108	0.7	24.8	34.6	7.45
			Recovery	160/82	72	137	3.2	109	0.8	24.8	38.9	7.44
J.S.			Control	120/86	68	131	4.3	105	1.0	24.1	41.5	7.39
F	21	1.90	Infusion	169/88	85	135	4.2	104	0.8	23.4	40.5	7.37
			Recovery	153/89	88	135	3.7	102	0.8	22.1	38.2	7.38
D.T.			Control	124/70	70	142	3.3	107	0.7	26.2	36.5	7.48
M	40	1.69	Infusion	156/70	80	143	3.8	108	0.7	26.9	36.5	7.48

See Table I for key to abbreviations.

*All values are the averages of two or more determinations. Ephedrine was administered intravenously during the "infusion" period. Subjects E. C. and J. S. had received 8 Gm. of ammonium chloride on the day prior to the study.

of the later phase of acid-base adjustments.[27] Explanation of the reduction in bicarbonate content at this time is not clear. Adjustment to this later and more prolonged displacement of acid-base balance is apparently made almost entirely by changes in ventilation. In the present study the calculated carbon dioxide tension of plasma usually fell and renal mechanisms for compensation were not apparent. Although tubular reabsorption of sodium and potassium increased at this time, as has been noted previously,[1-3] these changes in tubular activity appeared to be independent of the accompanying systemic metabolic alterations. The tubular response to l-norepinephrine was unmodified when, as a result of an infusion of sodium bicarbonate, plasma bicarbonate content increased rather than diminished and the plasma became more alkaline. Moreover, tubular exchange of hydrogen (as titratable acid) and ammonium ions was not accelerated during the infusion of norepinephrine, and sodium and potassium returned to the circulation largely as chloride salts, a transfer which, if prolonged, would have tended to perpetuate rather than alleviate the accompanying reduction in plasma bicarbonate content. However, in some subjects bicarbonate-obligated cation was reabsorbed in increased amounts as well, and displacement of acid-base equilibrium was thereby minimized. This may account in part for the absence of disturbances of acid-base balance in clinical circumstances, such as pheochromocytoma, in which prolonged or excessive adrenal medullary hormonal activity might be expected, although other corrective adjustments may also occur which limit not only this disturbance but the retention of electrolytes as well.[1]

The factors which determine the relative rates of chloride and bicarbonate reabsorption are obscure. The availability of anion did not appear to condition tubular activity, although in subjects who had received ammonium chloride the relative abundance of chloride in tubular urine, from which practically all bicarbonate-obligated cation was extracted, resulted in the reabsorption of cation almost entirely as chloride salts. In part, the reabsorption of bicarbonate-obligated cation appeared to be independent of the action of l-norepinephrine. In many instances changes in the excretion of bicarbonate paralleled changes

ON ACID-BASE EQUILIBRIUM AND URINE COMPOSITION*

	URINE									
GFR (ML./MIN.)	V (ML./MIN.)	pH (units)	Na (μEq/MIN.)	K (μEq/MIN.)	Cl (μEq/MIN.)	PO_4 (μM/MIN.)	HCO_3 (μM/MIN.)	pCO_2 (mM./Hg)	T.A. (μEq/MIN.)	NH_4 (μEq/MIN.)
108	3.8	5.17	125.6	70.5	177.6	17.1	0.7	43.0	25.5	27.0
131	8.8	5.51	186.4	52.8	217.5	17.4	3.1	44.0	28.8	40.9
129	6.8	5.29	141.0	39.8	163.0	17.0	1.3	39.7	27.1	33.3
113	3.5	4.71	197.0	85.9	300.0	13.6	0.2	33.4	31.2	36.8
96	4.3	4.69	168.0	70.0	184.0	13.6	0.3	39.0	27.6	30.9
117	6.9	4.95	148.0	59.7	213.7	16.2	0.6	37.1	31.8	35.4
69	6.9	6.56	158.8	61.8	144.1	8.8	26.2	42.8	10.0	18.1
76	7.8	6.45	211.0	45.4	179.1	10.1	26.7	49.9	11.7	20.2

in urine volume and were independent of alterations in sodium and potassium output. This phenomenon has been described previously[12, 19] and has been attributed to mixing of tubular urines of varying pH and buffer content.[12]

The site within the nephron of the accelerated tubular transfer of electrolyte is unknown. The data obtained in the present study are consistent with either proximal or distal tubular reabsorption, or both. However, tubular exchange mechanisms at either site appeared to play little role, unless reabsorption of bicarbonate-obligated cation is considered to occur in large part as a result of tubular cation exchange.[28] The failure of urinary acidity to change consistently may be attributed to the relatively constant excretion of titratable acid, to the inconstant alteration in bicarbonate output, and to the buffering capacity of the urine.

The mechanism of the electrolyte retaining activity of the adrenal medullary hormones was not clarified by the present study. This response appears to be independent of systemic acid-base adjustments and, as previous studies have demonstrated,[4] it is not mediated through secondary hormonal or neural pathways. Moreover, the different urinary electrolyte patterns elicited by ephedrine and l-norepinephrine, as demonstrated in this and previous investigations,[4] suggest that nonspecific pressor activity does not play a role. There remains the possibility, not tested in the present study, that local intrarenal circulatory adjustments and reduction in renal blood flow to tubular tissue elicit the retention of sodium and potassium chloride and bicarbonate or that the adrenal medullary hormones condition this response directly through alteration of tubular cellular metabolism. These views have been considered previously.[4]

SUMMARY

L-norepinephrine induced a reduction in plasma bicarbonate content with no change in pH when administered intravenously to normal human subjects. Renal tubular reabsorption of sodium, potassium, and chloride and of bicarbonate-obligated cation increased without consistent change in inorganic phosphate or ammonium excretion or in urinary pH or titratable acid excretion. These changes were observed in subjects premedicated with ammonium chloride and were unmodified during an infusion of sodium bicarbonate which progressively

increased plasma bicarbonate content. These results suggest that alterations in urine formation elicited by l-norepinephrine were independent of the accompanying displacements of plasma acid-base balance. Ephedrine sulfate elicited similar but less constant changes in plasma acid-base composition, but tended to depress rather than augment tubular electrolyte reabsorption.

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USE OF METHYLENE BLUE AS AN INDICATOR FOR ARTERIAL DILUTION CURVES IN THE STUDY OF HEART DISEASE

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MULTIPLE recordings of indicator-dilution curves following selective injections of dye into various sites in the central circulation have become an indispensable part of diagnostic cardiac catheterization procedures performed in this laboratory.[1-4] Their greatest usefulness has been in the detection, localization, and quantitation of intracardiac shunts in patients with congenital cardiac malformations.[3, 5, 6] Recently, dye-dilution curves have also found application in the diagnosis of acquired heart disease.[7-9]

Frequently, in order to allow a definitive diagnosis to be made during diagnostic right or left heart catheterization, multiple arterial dilution curves are recorded following injection of dye into various sites in the heart and great vessels. Evans blue dye (T-1824), the indicator commonly used, has the disadvantage of doses exceeding approximately 0.5 mg. per kilogram[10] which, in addition to causing bluish discoloration of the skin and mucous membranes when given in the cosmetic effect limiting the number of curves which may be performed, interferes with determination of blood oxygen saturation by oximetry and with the clinical evaluation of cyanosis, as for example, after cardiac operations.

To avoid this bluish discoloration it was decided to reinvestigate the use of methylene blue as an indicator in dye-dilution curves, this substance having been previously used for this purpose[11, 12] and found not to produce bluish discoloration in this same dosage range.

Evans blue and methylene blue are both aniline dyes. Evans blue (T-1824), a triphenyl methane dye which has a molecular weight of 960.83, has been widely used for blood-volume studies.[13-15] It can be quantitatively determined in blood spectrophotometrically in accordance with the Lambert-Beer law. Methylene blue or tetramethyl-thionine chloride, which has a molecular weight approximately one third as great as that of T-1824 (373.90), on the other hand, cannot easily be accurately quantitated in this fashion, since it exhibits the so-called spectral anomaly in aqueous solution, that is, deviation from the Lambert-Beer law, for reasons which are as yet not completely explained but in which reversible polymerization of the dye molecules is generally agreed to play a dominant role.[16-18]

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Fig. 1 compares the light transmission by aqueous solutions of both Evans blue* and methylene blue† dyes in equal concentrations of 5 mg. per liter. This graph also shows the sensitivity of the red photocell-filter assembly, the actual detecting element of the earpiece or cuvette oximeter, for changes in the amount of oxyhemoglobin and reduced hemoglobin or for changes in dye concentration. Since the peak sensitivity of the red photocell-filter assembly is at approximately 640 mμ, it is apparent that methylene blue is photometrically superior to Evans blue as an indicator for detection by oximetry. In this regard, it should be remembered that in similar concentrations of these dyes there are approximately three times as many molecules of methylene blue as of Evans blue. Using the values for optical density obtained spectrophotometrically, the molecular extinction coefficients (Emol) for aqueous solutions (5 mg. per liter) of Evans blue and methylene blue were calculated to be 40,000 and 60,000, respectively, at a wave length of 640 mμ.

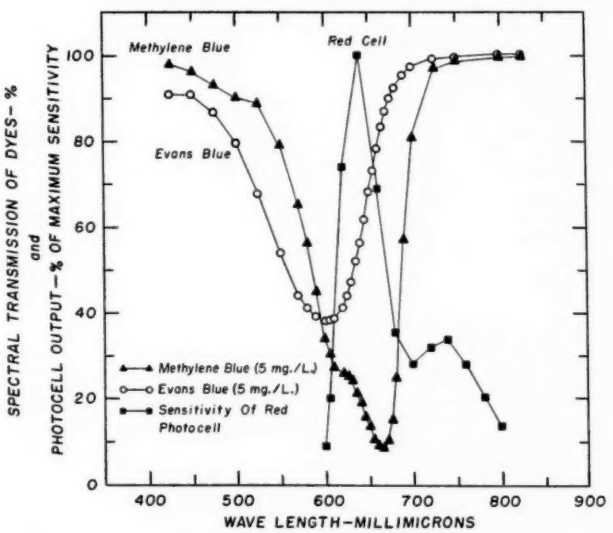

Fig. 1.—Comparison of light transmission of aqueous solutions (5 mg./L.) of Evans blue and methylene blue dyes and the relative spectral sensitivity of the red photocell-filter assembly of the oximeter. Note that at the peak sensitivity of this photocell (640 mμ) the methylene blue solution absorbs more light than Evans blue, transmitting approximately 20 per cent of the light of this wave length as compared to 55 per cent transmission for Evans blue.

METHODS

Dilution patterns were recorded simultaneously by earpiece and cuvette oximeters‡ following the sudden single injection of indicator using a technique previously described.[1] Successive injections into the same site in the central circulation of equal doses of both methylene blue and Evans blue dyes 3.3 to 21.1 minutes (average: 5.7) apart were performed in 12 normal persons and in 28 patients with heart disease. At times the recording of the methylene blue curve preceded that of the T-1824 curve, while at other times the order was reversed. The dosage of dye ranged from 0.1 to 0.3 mg. per kilogram (mean: 0.2 mg. per kilogram). To minimize interference in recording of the curves due to fluctuations in arterial blood oxygen saturation (reduced hemoglobin absorbs light of the same wave length as the dyes), most of the dilution curves were recorded with the normal subjects

*Warner-Chilcott Laboratories, New York, N. Y.
†Central Scientific Company, Chicago, Ill.
‡Manufactured by the Waters Corporation, Rochester, Minn.

and patients breathing 100 per cent oxygen. In specific instances dye curves were recorded with the patients breathing room air in order to permit subsequent determination of the effect of breathing high concentrations of oxygen on the magnitude of the intracardiac shunt[19] and also to see if methylene blue behaves differently under these two conditions. Sudden single injections of indicator were made into the right or the left pulmonary artery, the main pulmonary artery, the right ventricle, the superior vena cava, and the inferior vena cava. The dilution curves following each injection were recorded simultaneously at the two ears and directly in arterial blood by having blood flow through a cuvette oximeter attached to an indwelling needle in a radial artery. An example of an original photographic recording is shown in Fig. 2.

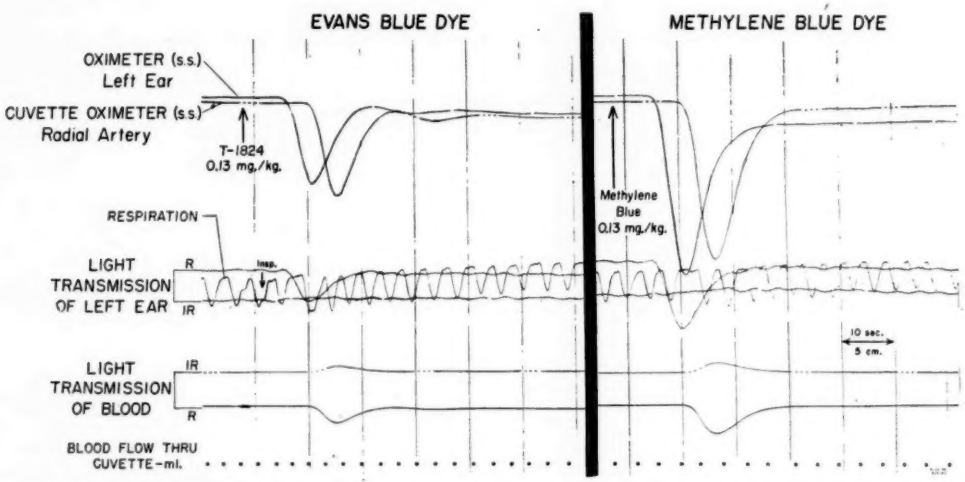

Fig. 2.—Original photographic record showing dilution patterns recorded simultaneously at the left ear and radial artery following successive injections of Evans blue and methylene blue dyes in equal doses (0.13 mg./Kg.) into the pulmonary artery of a healthy subject (man, 30 years old). The arrow indicates the instant of dye injection; subject breathing 100 per cent oxygen, in this and succeeding figures. In the methylene blue curves in contrast to Evans blue, note the greater peak deflection, the absence of a systemic recirculation peak, and the almost complete disappearance of detectable methylene blue from arterial blood within 60 seconds after injection, as contrasted to retention of an appreciable amount of the dye in the ear at this time.

In order to compare the contours of the curves obtained using the two different dyes in healthy subjects and in patients without intracardiac shunts, the peak deflection (PC), the deflection at the point of least concentration (LC), and that at the peak of the systemic recirculation (MRC),[20] as well as the deflection 30 seconds after the peak deflection, were measured in the Evans blue dye curves and at points corresponding in time on the associated methylene blue dye curves (Fig. 3). Similar measurements were made in the curves recorded simultaneously at the ears.

In curves recorded in patients with left-to-right intracardiac shunts the peak deflection (PC) and the deflections at 10-, 20-, and 30-second intervals after the peak were measured in a similar fashion in the T-1824 and the methylene blue curves recorded both directly in arterial blood (cuvette oximeter) and at the ears (Fig. 4). These measurements permit a comparison of the effect of the presence of a left-to-right shunt on the dilution patterns produced by the two different dyes, since a prolongation of the disappearance slope in Evans blue curves is a characteristic finding in patients with a hemodynamically significant left-to-right shunt.[6]

In curves obtained in patients having right-to-left shunts, the formula of Swan and co-workers[5] was applied to compare the values for the right-to-left shunt derived from successive T-1824 and methylene blue dilution curves.

Because it became evident from the recorded arterial dilution curves that the methylene blue dye was rapidly leaving the circulation and since alterations in the simultaneously recorded ear oximeter curves suggested the deposition of a portion of the dye in the tissues on its first circulation, it was decided to follow the changes in both T-1824 and methylene blue dye concentration in "bloodless tissue" (the pressurized ear) using an ear oximeter and applying the technique of Connolly and Wood.[10] The relative dye content of "bloodless" tissue was determined by inflating the pressure capsule of the ear oximeter immediately prior to, and on an average of, 2.67 minutes (range: 1.58 to 5.01 minutes) after the injection into the central circulation of T-1824 and 2.36 minutes (range: 1.42 to 4.01 minutes) after the injection of an equal dose of methylene blue.

RESULTS: COMPARISON OF CONTOURS OF METHYLENE BLUE AND EVANS BLUE DYE CURVES

A. Normal Subjects.—The peak deflections of the arterial dilution curves recorded at the radial artery in 12 healthy subjects following the injection of methylene blue, which amounted to 15.3 cm. (10.5 to 21.8), were 1.9 (1.5 to 2.2) times as great as those recorded following injection of equal doses of T-1824 (Table I). The appearance times and the times at which the peak deflection

TABLE I. DILUTION PATTERNS AT THE RADIAL ARTERY OF 12 NORMAL SUBJECTS AFTER SUCCESSIVE INJECTIONS OF EQUAL DOSES OF EVANS BLUE AND METHYLENE BLUE INTO THE PULMONARY ARTERY OR SUPERIOR VENA CAVA

	DEFLECTION AT:			
		LC	MRC	30 SECONDS AFTER PC
	PC (CM.)	AS PER CENT OF PC		
Evans blue	8.2	9.6	20.1	16.0
Methylene blue	15.3	8.0	4.3	2.9
Mean difference	−7.1	1.6	15.8	13.1
Range	−3.5 to −11.8	−4.2 to 6.1	13.7 to 19.9	7.5 to 19.0

PC = peak concentration; LC = least concentration; MRC = maximal recirculation concentration.

(PC) occurred were significantly earlier (0.8 second; range: 0.3 to 3.4 seconds) after injection of methylene blue than after injection of Evans blue. The methylene blue and Evans blue curves returned toward the base line at nearly similar times (T-1824 curves, time of occurrence of LC: mean, 13.2 seconds after PC; range, 10.0 to 17.0 seconds), the deflections at LC amounting to fairly similar percentages of their peak concentrations, comprising 8.0 per cent (3.8 to 11.4) and 9.6 per cent (2.4 to 17.0), respectively, of the peak deflections for the two dyes. Nevertheless, whereas the methylene blue curves steadily declined toward the base line for the remainder of their course, evidence of systemic recirculation, when present, being quite small, the T-1824 arterial blood and ear oximeter curves were characterized by the presence of a systemic recirculation peak of considerable magnitude which occurred at approximately 21.6 seconds (15.5 to 27.6 seconds) after PC (Table I and left panel of Fig. 3). At the height of the systemic recirculation (MRC) in the Evans blue curves the deflection produced directly in the arterial blood by T-1824 was still 20.1 per cent (16.3 to 24.5) of peak deflection, while, at a similar time interval from the peak, the methylene blue curves had diminished to a deflection

amounting to only 4.3 per cent (1.4 to 7.0) of their peak deflection. This difference in the concentrations of the two dyes in arterial blood was even more striking at a time 30 seconds after the peak concentration, when the methylene blue deflection had fallen to only 2.9 per cent (0.9 to 4.8) of peak deflection while the deflection produced by Evans blue still comprised 16.0 per cent (10.4 to 21.0) of its earlier peak deflection, a value which in the case of Evans blue is related to the blood volume of the individuals studied[1] (Table I and Fig. 3). This difference in concentration in arterial blood of the two dyes could indicate the occurrence of one of three possibilities: namely, that methylene blue is rapidly and completely reduced in the blood stream to its colorless leuko form, that the dye is lost from the blood stream to the tissues on its first passage through the circulation, or that both of these events are taking place simultaneously.

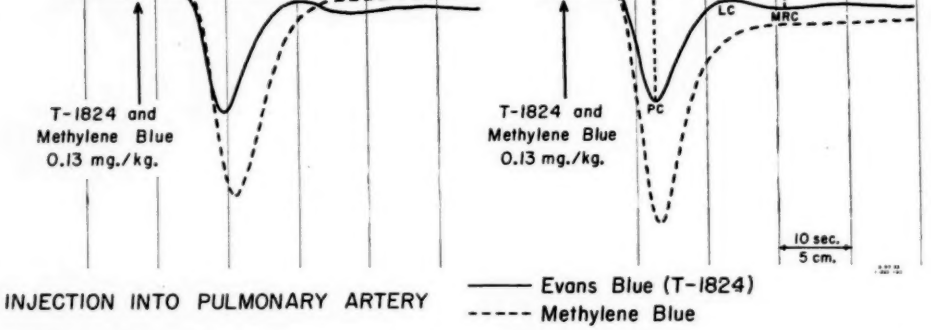

Fig. 3.—Arterial dilution curves recorded simultaneously by cuvette and ear oximeters following successive injections of Evans blue (solid lines) and methylene blue (dotted lines) dyes superimposed to facilitate comparison of their contours. The subject was a healthy man, 30 years of age. On the left are shown the curves recorded directly in arterial blood and on the right the ear oximeter curves. The Evans blue curves recorded directly in the blood and at the ears are similar, showing the usual pattern with a definite systemic recirculation peak and a plateau-equilibrium concentration which is related to the blood volume. In the methylene blue curves, in addition to the greater peak deflections and absent systemic recirculation peaks, note the rapid disappearance of the dye to almost zero concentration in arterial blood within seconds after injection, while the earpiece curve at this same time still shows the presence of a considerable amount of the dye in the ear (see text).

Evidence that methylene blue rapidly passes from blood to tissue was obtained from the dilution curves simultaneously recorded at the ears (right panel of Fig. 3). While the peak deflection of the ear oximeter curves recorded after injection of methylene blue again was greater than that after injection of T-1824 (mean value 2.1 times as great; range: 1.4 to 2.6), the amount of dye detected at the ear for the remainder of the methylene blue curve compared much more closely to that for the Evans blue curve than was the case directly in arterial blood. Thirty seconds after the peak concentration, at a time when the methylene blue content detectable directly in arterial blood was reduced to only 2.9 per cent of the peak, the methylene blue deflection at the ears still amounted to 17.1 per cent of the peak deflection as compared to 19.1 per cent for T-1824 (Table II). With the knowledge obtained from

the cuvette oximeter curves recorded directly in arterial blood that a relatively much smaller amount of methylene blue could be detected in the blood stream by this time, the conclusion that at least a portion of the methylene blue dye was entering the tissues (including ear tissue) during its first circulation seemed reasonable. Also supporting this view was the finding that in methylene blue curves recorded in normal subjects the systemic recirculation peak was either absent or markedly reduced and also the failure on several occasions to detect any recirculation of methylene blue dye at the ears or radial artery following the continuous, constant-rate injection of relatively large doses of this dye (20 mg. per minute) into the descending aorta.[21] Both of the latter findings, however, could also be explained by rapid reduction of the dye in blood to its colorless form.

TABLE II. DILUTION PATTERNS AT THE EAR OF 12 NORMAL SUBJECTS AFTER SUCCESSIVE INJECTIONS OF EQUAL DOSES OF EVANS BLUE AND METHYLENE BLUE INTO THE PULMONARY ARTERY OR SUPERIOR VENA CAVA

	DEFLECTION AT:			
	PC (CM.)	LC	MRC	30 SECONDS AFTER PC
		AS PER CENT OF PC		
Evans blue	6.1	12.3	22.1	19.1
Methylene blue	12.8	20.5	17.8	17.1
Mean difference	−6.7	−8.2	4.3	2.0
Range	−1.2 to −12.2	−1.8 to −17.1	−9.6 to 15.5	−17.7 to 14.4

PC = peak concentration; LC = least concentration; MRC = maximal recirculation concentration.

TABLE III. ABSENCE OF EVANS BLUE AND PRESENCE OF METHYLENE BLUE IN "BLOODLESS" EAR TISSUE IN 23 CASES APPROXIMATELY 2.5 MINUTES AFTER SUCCESSIVE INJECTIONS OF EQUAL DOSES OF THESE DYES

	OPTICAL DENSITY RATIO (D_R/D_{IR})		
	BEFORE INJECTION	1.4 TO 5.0 MINUTES AFTER INJECTION	DIFFERENCE
Evans blue	1.26 ± 0.02*	1.26 ± 0.02	0.00 ± 0.002
Methylene blue	1.25 ± 0.02	1.27 ± 0.02	0.02† ± 0.002

*The numbers following the ± signs are the standard errors of the mean values.
†Statistically significant; p value < 0.001.

Direct evidence for the rapid deposition of methylene blue in the tissues was obtained by spectrophotometric study of the light transmission of "bloodless" ear tissue. Optical density ratios (D_R/D_{IR}) of the "bloodless" ear tissue, calculated immediately before and at a mean time interval of 2.67 minutes (1.58 to 5.01 minutes) after injection of T-1824 into 23 healthy subjects and patients with heart disease, were not significantly different ($p < 0.9$). This confirms the previous findings of Connolly and Wood,[10] who also found no evidence of deposition of dye in the pinna of the ear or webs of the fingers immediately after injection of T-1824.

However, 2.36 minutes (1.42 to 4.01 minutes) after injection of an equal dose of methylene blue dye into the same 23 persons, the optical density ratios of the "bloodless" ear tissue were significantly increased over readings obtained before the injection ($p < 0.001$) (Table III). This corroborates the

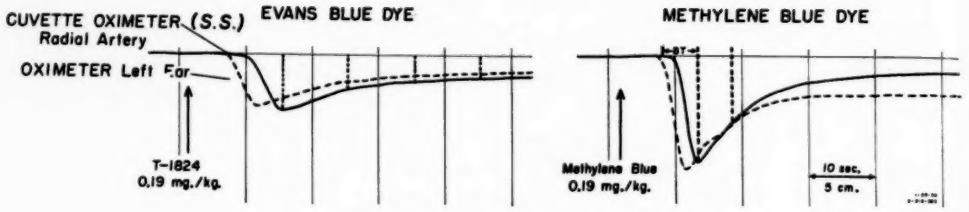

Fig. 4.—Comparison of arterial dilution curves following successive injections of Evans blue and methylene blue dyes (0.19 mg./Kg.) into the pulmonary artery of a patient (woman, 48 years of age) with a large intracardiac left-to-right shunt (80 per cent of pulmonary flow) via an atrial septal defect. Solid lines: direct arterial blood (cuvette oximeter) curves; dashed lines: ear oximeter curves. Note that the peak deflections of both curves are considerably smaller than in the curves recorded in the normal subject (Fig. 3), who actually received a proportionately smaller dose of dye. Note also the relatively greater peak deflection (PC) and the considerably faster decline in peak concentration in the methylene blue curve than in the Evans blue curve. Vertical dashed lines in the left panel indicate on the T-1824 curve the points at which deflections were measured in left-to-right shunt curves, that is, PC and the deflections at 10, 20, and 30 seconds after the peak concentration. Dashed lines in the right panel indicate points at which deflections were measured for calculation of the disappearance slope ratio. BT = build-up time on the cuvette curve.

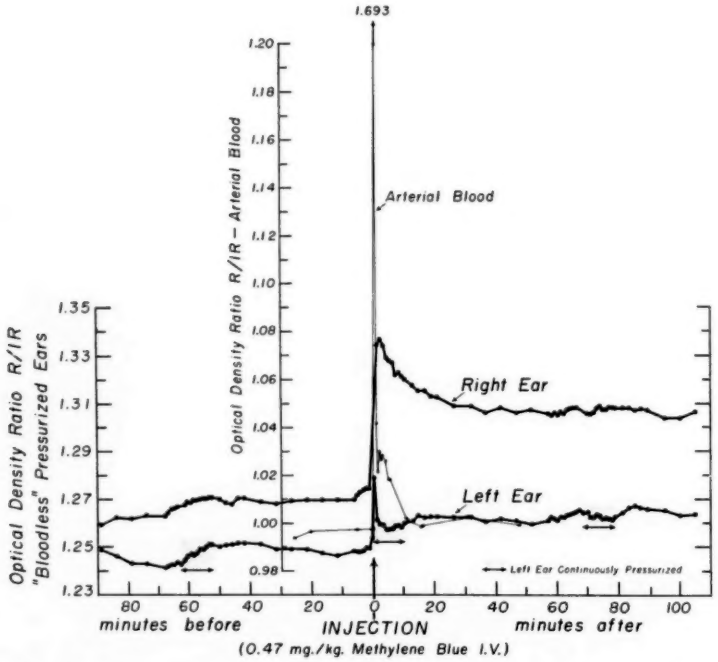

Fig. 5.—Effect of exclusion of blood from the ear tissue in the optical pathway of the ear oximeter on the pickup of methylene blue by this tissue during and after intravenous injection of the dye into a healthy subject (man, 38 years of age). The figure shows a comparison of the changes in apparent concentration of methylene blue in arterial blood and in "bloodless" (pressurized) ear tissue. Blood flow was excluded from the tissue in the optical path of the oximeter on the left ear for approximately 10 minutes beginning just prior to injection of the dye, while in the right ear blood flow to the tissue in the optical path of the oximeter was unimpeded during this period except during the 30-second periods required to measure the relative concentration of the dye in the pressurized ("bloodless") tissue of this ear. Note (1) the immediate high concentration (increase in optical density ratio) of methylene blue in arterial (radial artery) blood following injection and its subsequent rapid disappearance, (2) the immediate detection of a considerable quantity of methylene blue in the "bloodless" tissue of the right ear, and (3) that the apparent pickup of dye in the "bloodless" tissue of the left ear (from which blood was excluded during the period of high concentration in arterial blood) was much less than in the right ear. The initial peak of apparent concentration in the pressurized left ear, simultaneous with the peak concentration in arterial blood, is due to the effect of reflected light from the tissue contiguous to the pressurized area from which blood flow could not be excluded. Similarly, the small persisting increment in the optical density of the left ear tissue following the dye injection is probably produced in the same manner by the dye taken up by the contiguous (nonpressurized) tissues.

suggestion from the simultaneous cuvette and earpiece oximeter dye-dilution curves indicating that the methylene blue dye was rapidly leaving the blood stream and at least a portion of it was being deposited in the tissues.

To further confirm this observation a series of experiments was carried out in which dilution curves of methylene blue were recorded simultaneously in radial artery blood and at both ears; the blood, however, was excluded from the optical path of one earpiece during the dilution curve by inflating its pressure capsule to 250 mm. of mercury. When this was done, the quantity of methylene blue subsequently detectable in the ear tissue from which blood was excluded during the dilution curve was strikingly less than in the ear tissue with unimpeded blood flow during and immediately after injection of methylene blue (Fig. 5).

To study transformation of the spectral transmission of the dye in blood in vitro, successive sudden injections of Evans blue and methylene blue dye were made into oxygenated fresh human blood (heparinized) contained in a moving Waring blendor from which the blood was being continuously sampled through a cuvette oximeter. Comparison of the resultant curves of dilution (Fig. 6) shows an apparent disappearance of the methylene blue dye in the blood, which occurred at a progressively slower rate after initial mixing of the dye and blood. This decrease in spectral absorption by methylene blue in blood may be due to the movement of dye into the cells and its intracellular reduction to the colorless leuko form.

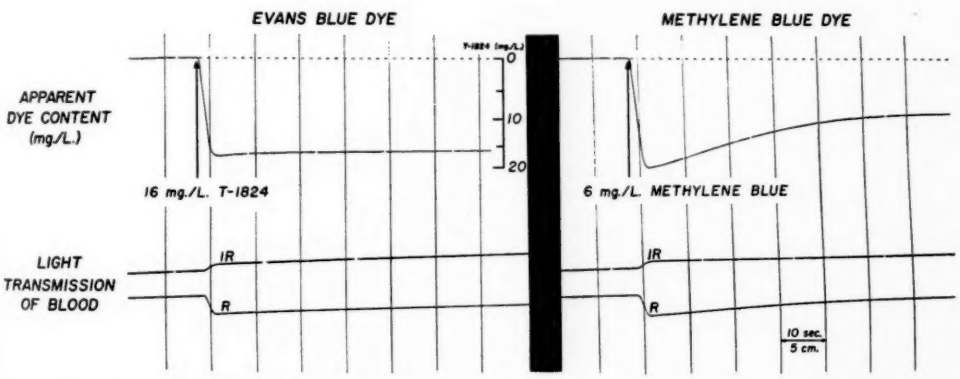

Fig. 6.—Original recording of apparent changes in concentration of Evans blue and methylene blue immediately after mixing of dye and blood in vitro (see text). Note that after a small "overshoot" (not instrumental) the recorded concentration of Evans blue in the mixture remains stable. In contrast, a rapid "disappearance" of methylene blue occurs, apparently due to decolorization of the dye in blood. The apparent concentration of methylene blue in the mixture decreased in 2 minutes to about 50 per cent of its initial value and remained approximately stable at this value for the duration of the observations (30 minutes).

B. Patients With Left-to-right Intracardiac Shunts.—Decrease in the magnitude of the peak deflection and prolongation of the disappearance slope associated with an absent systemic recirculation peak in T-1824 arterial dilution curves have become recognized as the hallmarks of the presence of a left-to-right shunt[6] (Fig. 4). These findings have also been noted in dilution curves recorded in patients with severe valvular insufficiency.[7-9] When compared to the mean peak deflections obtained in the normal subjects with the

two dyes, the diminution in magnitude of the peak deflections in 10 patients with left-to-right shunts was similar in the methylene blue and T-1824 curves, the mean peak concentrations amounting to 66.1 and 63.5 per cent, respectively, of the mean values for peak concentration obtained in the normal subjects. The degree of prolongation of the disappearance slope, however, was considerably less in the curves recorded after injection of methylene blue than after injection of Evans blue. Comparison of the deflections detected directly in the arterial blood 10, 20, and 30 seconds after the peak deflection in successive methylene blue and Evans blue curves in these 10 patients with left-to-right shunts revealed deflections amounting to 51, 41, and 34 per cent of peak deflection in the Evans blue curves at these times compared to concentrations of 31, 16, and 11 per cent of peak deflection in the curves recorded following injection of methylene blue (Table IV). These figures, however, do indicate the presence also of definite prolongation of the disappearance slope of the methylene blue curves in patients with left-to-right shunts compared to curves obtained in normals in whom at similar times, dye concentrations were approximately one-fourth of these values (Table I). Gross inspection of the curves revealed no difference in the behavior of methylene blue as an indicator when the patients were breathing air or oxygen.

TABLE IV. DILUTION PATTERNS AT THE RADIAL ARTERY OF 10 PATIENTS WITH INTRACARDIAC LEFT-TO-RIGHT SHUNTS AFTER SUCCESSIVE INJECTIONS OF EQUAL DOSES OF EVANS BLUE AND METHYLENE BLUE INTO THE PULMONARY ARTERY OR SUPERIOR VENA CAVA

		10 SECONDS AFTER PC	20 SECONDS AFTER PC	30 SECONDS AFTER PC
	PC (CM.)	AS PER CENT OF PC		
Evans blue	5.2	50.6	40.6	34.0
Methylene blue	10.1	31.0	16.4	10.9
Mean difference	−4.9	19.6	24.2	23.1
Range	−11.4 to 0.3	1.4 to 28.8	12.5 to 41.3	11.4 to 41.2

PC = peak concentration.

TABLE V. COMPARISON OF DISAPPEARANCE SLOPE RATIOS CALCULATED FROM SUCCESSIVE EVANS BLUE AND METHYLENE BLUE ARTERIAL DILUTION CURVES IN 12 NORMAL SUBJECTS, IN FOUR PATIENTS WITH STENOTIC VALVULAR HEART DISEASE AND IN 12 PATIENTS WITH INTRACARDIAC LEFT-TO-RIGHT SHUNTS

	DISAPPEARANCE SLOPE RATIO IN:		
	NORMAL SUBJECTS	PATIENTS WITH STENOTIC VALVULAR HEART DISEASE	PATIENTS WITH INTRACARDIAC LEFT-TO-RIGHT SHUNTS
Evans blue	0.30* (0.21–0.38)†	0.32 (0.25–0.40)	0.65 (0.44–0.88)
Methylene blue	0.26 (0.16–0.34)	0.26 (0.15–0.37)	0.50 (0.35–0.72)

*Mean value.
†Range.

To quantitate the disproportionate prolongation of the disappearance slope caused by a left-to-right shunt, the so-called disappearance slope ratio was devised. This ratio is calculated by dividing the deflection occurring at a time equal to the build-up time (BT)[20] after the peak deflection by the peak deflection (PC): Disappearance slope ratio = (Deflection BT seconds after PC) ÷ PC (Fig. 4, right panel).

In Fig. 7 the disappearance slope ratios for the successive Evans blue and methylene blue curves recorded at the radial artery are compared with the magnitudes of the left-to-right shunts measured in 12 patients during the same cardiac catheterization procedure. A fairly good correlation between the size of the left-to-right shunt and the disappearance slope ratio is evident both for the Evans blue and for the methylene blue arterial dilution curves. The mean values and ranges for the disappearance slope ratios determined from the successive dilution curves recorded in normal subjects and in the cardiac patients with and without left-to-right shunts are given in Table V. Patients with valvular heart disease in whom the presence of valvular insufficiency was found at operation or necropsy or in whom there was a strong clinical suspicion of the presence of valvular insufficiency were excluded. As is evident both from Table V and from Fig. 7, there was no overlap of normals and left-to-right shunt patients when this ratio was calculated either from the T-1824 curve or from the associated methylene blue curve. As expected, owing to the rapid loss of methylene blue from blood, ratios calculated from methylene blue curves were systematically smaller than those for Evans blue.

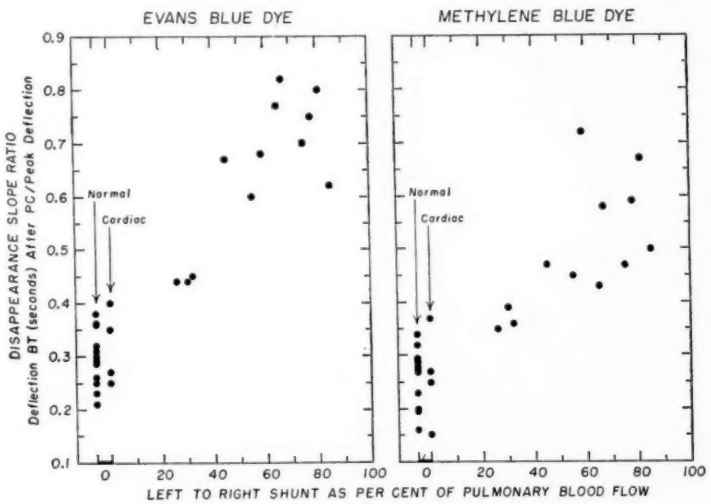

Fig. 7.—Relation of left-to-right shunt to disappearance slope ratio calculated from successive Evans blue and methylene blue dilution curves recorded at the radial artery in 12 patients with intracardiac left-to-right shunts. Note the fairly good correlation between the size of the left-to-right shunt (measured at cardiac catheterization) and the disappearance slope ratio of both the Evans blue (left panel) and methylene blue (right panel) dilution curves. The disappearance slope ratios obtained in 12 normal subjects and 4 patients with stenotic valvular heart disease (in whom both an intracardiac shunt and significant valvular insufficiency had been excluded) are plotted along the ordinate.

C. Patients Without Intracardiac Shunts.—In the nine cardiac patients without significant intracardiac shunts studied, comparison of the successive methylene blue and Evans blue arterial dilution curves yielded results which would be expected on the basis of the foregoing data. In 4 patients who had severe mitral or aortic stenosis (without significant insufficiency) the T-1824 curves were of the typical so-called failure type, that is, the curves were shallow and all of the time components were markedly but symmetrically prolonged

(Fig. 8). Accompanying methylene blue curves revealed peak concentrations which were 1.9 (1.4 to 2.5) times as great as those after T-1824 and similarly displayed a prolongation of all of the time components. The disappearance slope of these curves, however, again was proportionately less prolonged than in the associated Evans blue curves, as was the case in the normal subjects and in patients with left-to-right shunts.

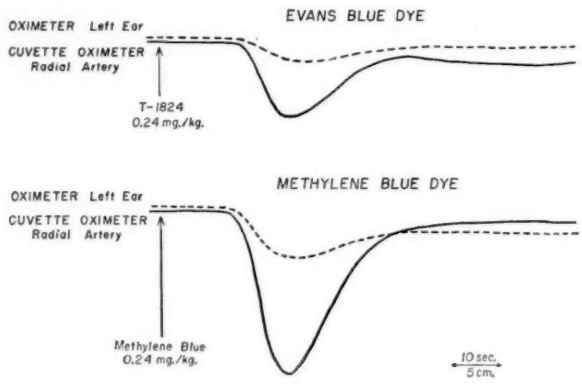

Fig. 8.—Comparison of arterial dilution curves following successive injections of Evans blue and methylene blue dyes into the pulmonary artery of a patient with stenotic valvular heart disease without an intracardiac shunt (man, 36 years of age; aortic stenosis). Note the prolongation of all of the time components of these curves, especially the prolonged systemic recirculation time of 52 seconds (normal: approximately 21 seconds). The systemic recirculation peak is evident only on the T-1824 curve recorded at the radial artery. The differences in amplitudes of the curves recorded at the ear and at the radial artery are due to the low sensitivity of the earpiece to dye in arterial blood which is encountered occasionally and occurred in this particular patient.

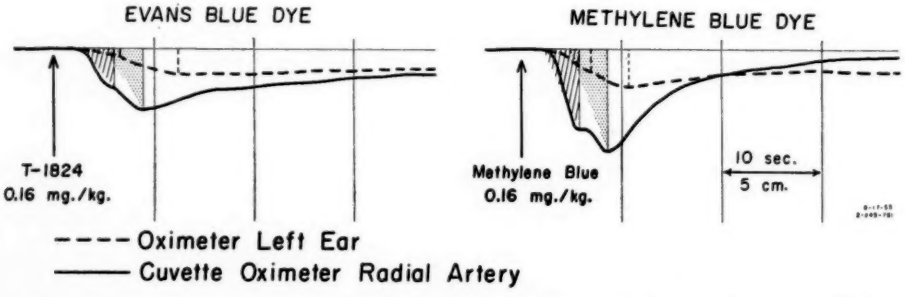

Fig. 9.—Comparison of arterial dilution curves recorded following successive injections of Evans blue and methylene blue dyes into the superior vena cava of a patient with an intracardiac right-to-left shunt (girl, 5 years of age; ventricular septal defect with pulmonary hypertension). The right-to-left shunt pattern consists of an early appearance of dye with a double hump contour, the first hump representing dyed blood which has passed through the defect directly into the systemic circulation to the sampling site and the second hump representing dyed blood which has followed the normal, longer pathway through the lungs. The magnitude of the right-to-left shunt is determined by dividing the area of the first or shunt triangle (slanted lines) by the sum of the areas of the two triangles shown.[5] Note the proportionately greater area of the initial triangle in the methylene blue curve, which calculated out to represent a 40 per cent right-to-left shunt compared to a 25 per cent shunt calculated from the Evans blue curve. This difference is due to loss of methylene blue in the lungs coupled with rapid decolorization in the blood (see text). Note also the decreased amplitude and relatively poor definition of the curves recorded at the ear; this is sometimes seen, especially in infants.[4]

D. Patients With Right-to-left Intracardiac Shunts.—In 9 patients with congenital heart disease in whom the presence of an intracardiac right-to-left shunt was detected during cardiac catheterization by means of dye-dilution curves and, in some of the cases, also from the arterial blood oxygen saturation

data, successive methylene blue and T-1824 curves were recorded at a mean time interval of 4.0 minutes (range: 3.1 to 4.8 minutes). In 3 of the patients the veno-arterial shunt was located at the atrial level and in the remaining 6 it was at the ventricular level.

Analysis of these curves was confined to a comparison of the magnitude of the calculated right-to-left shunts using the formula of Swan and associates[5] (Fig. 9). A similar percentage shunt would be expected if the areas of the primary and secondary deflections in curves recorded following the injection of each of these dyes maintained a similar proportion to each other.

From Table VI it can be seen that in the patients with moderately sized right-to-left shunts, all of whom had an interventricular communication, methylene blue curves tended to exaggerate the magnitude of the shunt (see also Fig. 9). In the 4 patients with atrial septal defect, all with small right-to-left shunts, the shunts calculated from the methylene blue and Evans blue dye curves were not significantly different.

TABLE VI. COMPARISON OF RIGHT-TO-LEFT SHUNTS CALCULATED FROM SUCCESSIVE DYE-DILUTION CURVES USING EVANS AND METHYLENE BLUE

RIGHT-TO-LEFT SHUNT AS PER CENT OF SYSTEMIC FLOW IN:					
FOUR PATIENTS WITH ATRIAL SEPTAL DEFECTS			FIVE PATIENTS WITH VENTRICULAR SEPTAL DEFECTS		
EVANS BLUE	METHYLENE BLUE	DIFFERENCE	EVANS BLUE	METHYLENE BLUE	DIFFERENCE
10	10	0	33	36	−3
			32	32	0
8	9	−1	29*	39	−10
10	11	−1	23	41	−18
			39	42	−3
			28*	50	−22
5	3	2	23	45	−22
			23*	31	−8

*Patient breathing room air; all other curves recorded with patient breathing 100 per cent oxygen.

The initial deflection of dilution curves recorded in the presence of a right-to-left shunt (Fig. 9) represents dyed blood which passes through the intracardiac defect directly to the systemic arteries and sampling site without traversing a capillary bed, while the second deflection represents dyed blood which has passed in the normal fashion through the capillary bed of the lungs. Therefore, if a significant amount of methylene blue disappeared from the blood stream during passage through the lungs a discrepancy in the estimate of veno-arterial shunts calculated from methylene blue and Evans blue dye curves would be expected.

The technique of simultaneous right and left heart catheterization[22] afforded an opportunity of obtaining direct evidence with regard to possible loss of methylene blue dye in the lungs. Dilution curves were recorded simultaneously at the radial artery and at the ear following successive injections of equal doses of T-1824 and methylene blue into the left atrium and the pulmonary artery. Dye injected into the pulmonary artery must traverse the pulmonary

capillary bed, while dye injected into the left atrium reaches the sampling site without passage through lung tissue. Also because of the longer distance to the sampling site, dye injected into the pulmonary artery remains in contact with the blood for a longer period than that injected into the left atrium, again giving a greater opportunity for disappearance of dye in the blood proper, as illustrated in Fig. 6. By comparing the ratio of the peak deflections recorded at the radial artery following the injections of identical doses of T-1824 (which is known to remain in the blood stream for a considerable period[10]) into the pulmonary artery and into the left atrium with the ratio obtained following the injection of methylene blue dye into these sites, an estimate of the average amount of dye disappearing during passage through the lungs is possible. In 5 such experiments, the amount of methylene blue dye "lost" in the lungs, as estimated by the foregoing method, was found to be 13 per cent (range: 1 per cent to 40 per cent).

DISCUSSION

In substituting methylene blue for Evans blue as indicator in arterial dilution curves an immediately apparent disadvantage is the inability to use methylene blue curves for cardiac output determinations, since this dye cannot easily be quantitated spectrophotometrically in blood. Methylene blue, however, finds considerable use as an indicator in diagnostic cardiac catheterization. In patients with left-to-right shunts it has been possible to estimate grossly the magnitude of the shunt from the Evans blue dilution curves.[6] Reorientation of the criteria used in this method of estimating the magnitude of left-to-right shunts is necessary when methylene blue is used, because of the (apparently) lesser degree of prolongation of the disappearance slope in the methylene blue curves. When this is done, a semiquantitative estimation of the magnitude of the left-to-right shunt is possible from the methylene blue curves of apparently the same degree of accuracy as when Evans blue is used. With regard to the detection of left-to-right shunts by means of dye curves it must be remembered that the systemic recirculation peak may be absent in methylene blue dilution curves recorded in normal subjects and that this deficiency cannot be regarded as an abnormal finding.

Because, milligram for milligram, it produces twice the deflection of T-1824, methylene blue has an advantage over T-1824 in the detection and localization of right-to-left shunts.

Another important function which methylene blue serves in the diagnostic applications of indicator-dilution curves is to permit the repetition of dilution curves, unsatisfactory for technical or other reasons, without fear of gross discoloration of the patient's skin, as would be the case were Evans blue the only available indicator.

Indigo carmine, which has recently been used in the performance of arterial dilution curves,[23] offers the possibility of overcoming some of the disadvantages of methylene blue. Because it is not lost from the blood stream before one circulation is completed, its dilution patterns can be used for determination of cardiac output, and for this same reason they resemble closely

curves recorded following injection of T-1824. Its relatively low spectral absorption in the region of 640 mμ requires the use of comparatively large doses of this substance for the satisfactory recording of arterial dilution curves by means of oximetry. However, this dosage does not produce appreciable discoloration of the skin.[24]

SUMMARY

The contours of dilution curves recorded simultaneously at the radial artery and at the ears following successive injections of equal doses of Evans blue and methylene blue were studied in 12 healthy subjects, 28 patients with congenital heart disease, and 9 patients with acquired heart disease.

1. Methylene blue in doses required for diagnostic purposes does not produce discoloration of the skin, as may occur following use of Evans blue.

2. In equal doses methylene blue produced a peak deflection which was approximately double that following injection of Evans blue; however, the systemic recirculation peak was diminished or absent.

3. Methylene blue was found to be largely lost from the blood stream on its first circulation and could be detected in bloodless ear tissue within seconds after its injection.

4. Determinations of dye content in bloodless (pressurized) ear tissue 2.5 minutes after intravenous injection of 0.2 (0.1 to 0.3) mg. per kilogram of Evans blue and of methylene blue revealed appreciable quantities of methylene blue but no detectable Evans blue in the tissue at this time.

5. In vitro experiments revealed rapid (approximately 50 per cent) diminution in spectral absorption within 2 minutes after the mixing of methylene blue dye and whole blood. The apparent concentration of dye in the mixture then remained approximately stable for the duration of the observations (30 minutes).

6. Appreciable apparent loss of methylene blue from the blood stream during its first passage through the pulmonary circulation was demonstrated.

7. In patients with left-to-right shunts a similar degree of correlation was demonstrated between the degree of disproportionate prolongation of the disappearance slope of Evans blue and methylene blue dilution curves and the magnitude of the shunt as determined by cardiac catheterization.

8. Methylene blue can be used in a similar fashion to Evans blue dye for the detection and localization of right-to-left shunts. Loss of methylene blue in the dye-blood mixture traversing the lungs may result in an overestimation of the magnitude of right-to-left shunts when this indicator is used.

9. It is concluded that methylene blue is a valuable adjunct to Evans blue for use as an indicator for recording of selective dye-dilution curves during diagnostic cardiac catheterization.

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THE METABOLIC EFFECTS OF PROGESTERONE IN MAN: DOSE RESPONSE RELATIONSHIPS

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SEVERAL aspects of the metabolic influence of administered progesterone in men and women have been described in recent publications by this group.[1-4] Confirming an isolated experiment performed by Abels and Dobriner,[5] it was shown that progesterone in a dosage of 50 to 100 mg. daily consistently induced a moderate increase in the net rate of protein catabolism. Kyle and Hess[6] have obtained similar results. This nitrogen dissipation probably results from a direct or indirect effect on several organs or tissues, since, with the exception of muscle, no single tissue could provide the amount of protein catabolized as a result of this treatment. The process is not mediated by the adrenals, the pituitary, or thyroid glands and does not require the presence of adrenal or pituitary secretions. Estrogen does not potentiate the response and, although anabolic itself, does not inhibit it.

Progesterone was also shown to possess a transient sodium and chloride dissipating influence in subjects with normal adrenal glands, and a more intense and protracted saluretic effect in desoxycorticosterone and cortisone-treated Addisonians. In one adrenal deficient patient progesterone did not affect sodium and chloride excretion when the adrenal insufficiency was treated with salt alone, but after cortisone and desoxycorticosterone had been given the usual intense salt loss was induced. It was accordingly suggested that this effect of progesterone was probably explained by the competitive inhibition at a renal level of adrenal salt retaining hormones.

The 50 and 100 mg. daily dosages of progesterone which were employed in the above studies are amounts which, judging from pregnanediol excretion studies, approximate the quantity of progesterone secreted during early pregnancy.[2] From the standpoint of gaining an appreciation of the importance of the metabolic effects of progesterone in human physiology it was also necessary to determine its influence at dosages comparable to the amounts secreted at other times. Accordingly the effects of 6.25 to 300 mg. per day have been studied in several subjects, thus covering the physiologic range from the progestational phase of the menstrual cycle to the last trimester of pregnancy.

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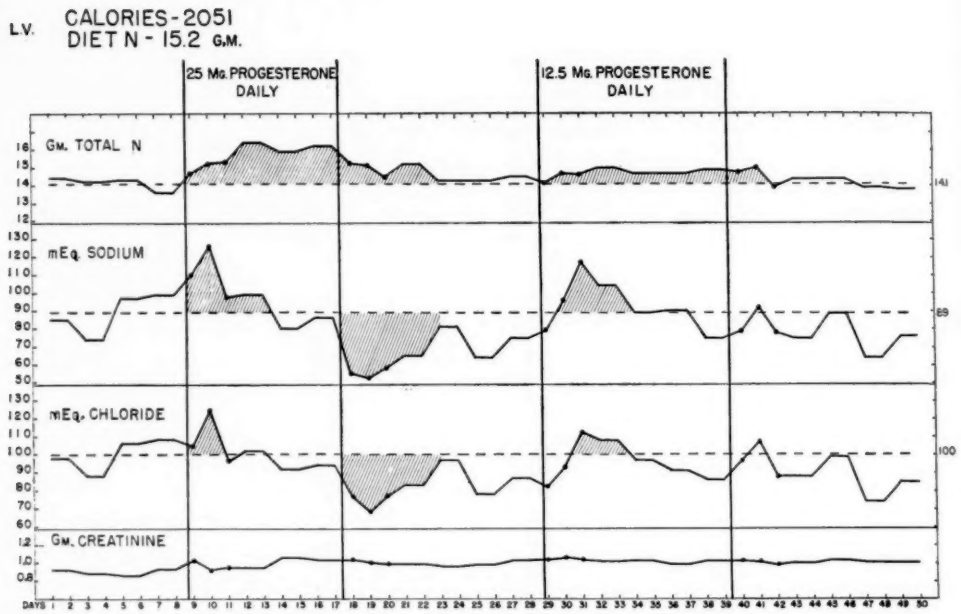

Fig. 1.—The effects of courses of 12.5 and 25 mg. of progesterone daily on the urinary excretion of total nitrogen, sodium, chloride, and creatinine in L.V., an arthritic man. Broken horizontal lines indicate the averages of pretreatment controls.

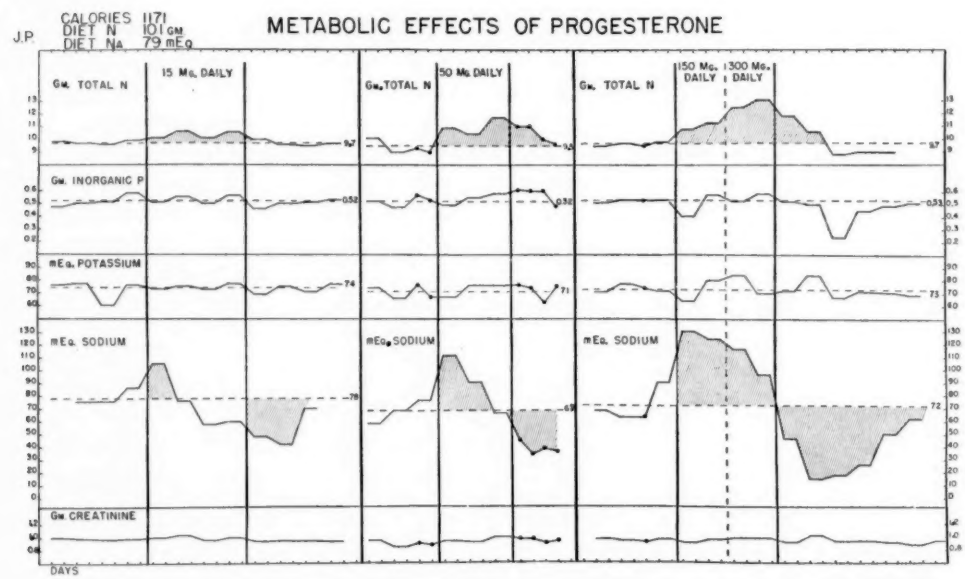

Fig. 2.—The effects of 15, 50, 150, and 300 mg. per day of progesterone on the urinary excretion of total nitrogen, inorganic phosphorus, potassium, sodium, and creatinine in J. P., the obese diabetic woman. Fasting and 4:30 P.M. postprandial blood sugars drawn during treatment varied from 112 to 168 mg. per 100 ml. and did not differ significantly from control levels. No glycosuria occurred at any time. Broken horizontal lines indicate the average of control values.

SUBJECTS

L. V., a 60-year-old man, was rather severely crippled by rheumatoid and degenerative arthritis. At the time these studies were performed he was afebrile and the rheumatoid arthritis was inactive. His basal metabolic rate (Mayo Foundation Standard) was +5 with basal calories of 1,460 per day.

L. S., a 53-year-old postmenopausal woman, was chronically undernourished because of functional anorexia. She weighed just 77 pounds. The basal metabolic rate was −10 with basal calories of 820. For the sake of brevity detailed data from this experiment are not included, but will be referred to in the text.

J. P. was a 56-year-old postmenopausal woman suffering from diabetes, obesity, and arteriosclerotic heart disease. When she was on a restricted diet no insulin was required, but when she ate freely, as was often her habit, she took as much as 80 units of insulin per day. During the studies recorded here no insulin was required.

R. B. was a 53-year-old obese man with mild arteriosclerotic heart disease. His metabolic rate was +7 with basal calories of 1,940 per day.

E. C. was a postmenopausal woman with chronic neurodermatitis of the thighs. Her metabolic rate was −15 with basal calories of 1,090 per day. Detailed data are not presented, but are commented upon in the text.

METHODS

All of the subjects resided in the metabolic unit of the hospital. Physical activity varied little from day to day. Dietary and fluid intake were maintained at a constant level in the usual fashion.[7] The obese subjects were on moderate reducing programs; the others were fed maintenance or supramaintenance diets. The progesterone, dissolved in sesame oil and 20 per cent benzyl benzoate, was administered intramuscularly in single daily injections for the smaller doses, but in 2 or 3 spaced injections for the 200 to 300 mg. daily dosages. Analyses were carried out on 24- or 48-hour urine pools, the completeness of collections being tested for by the performance of creatinine determinations. As in the past a metabolic effect was assumed to have occurred when treatment was associated with a significant deviation from the base-line values of the urinary constituent determined. Complete balances were not performed. The fecal excretion of nitrogen, inorganic phosphorus, sodium, potassium, and chloride was assumed to vary insignificantly from day to day. None of the patients had gastrointestinal disease. Total urinary nitrogen, inorganic phosphorus, and creatinine were determined as previously described.[7] Sodium and potassium were measured by means of the Perkin Elmer flame photometer using a lithium internal standard. Pregnanediol was determined by a modification of Stimmel's technique.[8]

RESULTS AND DISCUSSION

In 3 subjects dosages of only 12.5 to 15 mg. per day of progesterone evoked a catabolic response. Although the effects were minimal, elevations in urinary nitrogen were consistent and systematically correlated with the time of progesterone administration. In L. V. (Fig. 1), the 12.5 mg. course induced a nitrogen elevation which averaged 0.6 Gm. daily. In J. P. (Fig. 2) and L. S. (data not shown), 15 mg. daily of progesterone resulted in average increases of 0.7 and 0.6 Gm. of nitrogen per day, respectively. In L. V., 6.25 mg. of progesterone per day failed to affect urinary nitrogen excretion, although the characteristic rise in basal body temperature was observed. It would thus appear that 12.5 mg. per day of progesterone approaches the minimum dosage which could detectably enhance protein catabolism in man.

Some gradation in the intensity of the catabolic response to progesterone was apparent as the dosage was increased to 25 and 50 mg. per day. In L. V. (Fig. 1) the daily nitrogen elevation averaged 1.7 Gm. during the 25 mg. daily course of progesterone, and in J. P. (Fig. 2) the nitrogen dissipation was increased to an average of 1.5 Gm. per day when 50 mg. was given. These subjects served as their own controls and the differences in response at the two dosage levels in each individual seem quite significant. Furthermore the 0.6 to 0.7 Gm. average elevation induced by 12.5 or 15 mg. of progesterone in these 3 experiments is distinctly less than the 1 to 2 Gm. increments observed as a result of the daily administration of 50 mg. per day of progesterone in over 20 published[2, 4] and unpublished experiments.

The elevation in urinary nitrogen tended to be maximal at a dosage of about 50 mg. per day. In some subjects, however, larger amounts of progesterone evoked a somewhat greater response. In a previously reported experiment[2] 100 mg. daily induced a nitrogen elevation of 1.7 Gm. per day in comparison with the 1.1 Gm. average increase which resulted from treatment with 50 mg. The average elevation induced by 50 mg. per day in J. P. (Fig. 2) was 1.5 Gm.; during the 4 days on 150 mg. the increase averaged just 1.4 Gm., but doubling the dosage in the succeeding 4 days was associated with a further increase to a level 3.0 Gm., higher than the control base line. On the other hand, in R. B. (Fig. 3) the nitrogen elevation induced by separate courses of 50, 200, and 300 mg. of progesterone per day were essentially the same, and in the postmenopausal woman, E. L. (data not shown), the nitrogen increments were also indistinguishable on these dosages. It may be concluded that even if these large quantities of progesterone should increase the catabolic process beyond that induced by 50 mg. per day, the urinary nitrogen elevation remains of the same order of magnitude, and the relatively extreme elevations which may be induced by large excesses of adrenal corticoids[9, 10] are not seen when as much as 300 mg. of progesterone is given each day. In considering this comparison, however, it should also be emphasized that when cortisone is given in dosages of 50 mg. per day or less, there is often no detectable catabolic response.

The influence of progesterone on urinary inorganic phosphorus and potassium in R. B. (Fig. 3) is unique in our experience. It has been stated[2] that when progesterone is given to individuals with normal adrenal glands the excretion of these constituents is not significantly affected. As a matter of fact, in about one-half of our experiments urinary potassium dropped approximately 7 meq. and inorganic phosphorus about 0.1 Gm. during the first two days of treatment. These shifts were more or less disregarded because they were trivial and inconsistent. In R. B. (Fig. 3), however, this prompt suppression in the excretion of both substances was conspicuous at all 3 dosages. Inorganic phosphorus dropped 0.24, 0.23, and 0.24 Gm. per day, and potassium 21, 12, and 14 meq. per day. The excretion of both then returned to the base line and rose above it in parallel fashion, returning to the control level several days after treatment had stopped. These shifts probably represent an

unexplained exaggeration of the dip in urinary potassium and inorganic phosphorus seen in some other subjects (Fig. 2; 150 mg. dose). As such they may be assumed to reflect a metabolic change involving both phosphate and potassium.

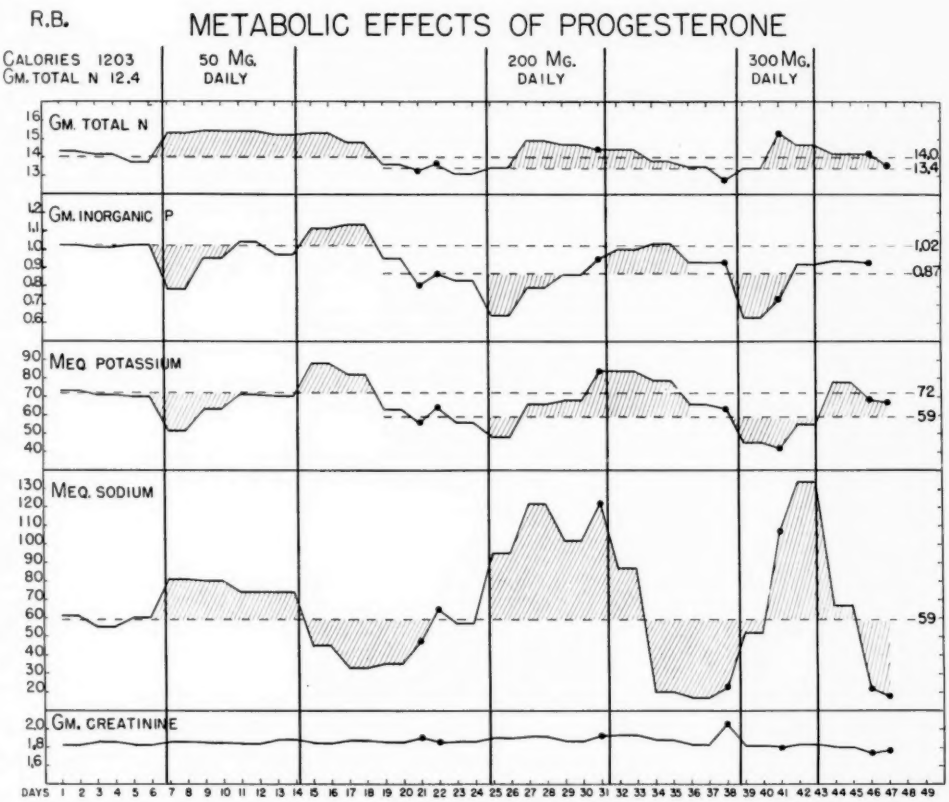

Fig. 3.—The effects of courses of 50, 200, and 300 mg. of progesterone each day on the urinary excretion of total nitrogen, inorganic phosphorus, potassium, sodium and creatinine in R. B., the obese man. Broken horizontal lines indicate the averages of control values. Two control levels are indicated in the excretion of total nitrogen, inorganic phosphorus, and potassium because the base line of these constituents dropped after the first treatment.

The dissociation of these parallel shifts in potassium and phosphorus from the rising curves of urinary nitrogen excretion suggests that whatever metabolic process is involved, it is relatively independent of protein catabolism. In some circumstances, i.e., anabolic processes induced by androgen,[7] the urinary inorganic phosphorus and potassium excretion closely paralleled total nitrogen excretion, indicating their storage with protein tissue. In some Addisonians[2] in whom a rather intense catabolism was induced by progesterone the urinary excretion of inorganic phosphorus and potassium rose with the total nitrogen, presumably reflecting the acceleration of protein tissue dissolution. The sharp initial decline in the excretion of inorganic phosphorus and potassium which is dramatized in R. B. (Fig. 3) seems to point to some

other influence of progesterone—perhaps an effect on carbohydrate metabolism. In this connection it should be noted that in the controlled diabetic subject J. P. (Fig. 2) progesterone did not induce glycosuria, and the fasting and postprandial blood sugars were not affected by treatment.

The intensity of the salt dissipating action of progesterone was very clearly enhanced by increasing the dosage of the hormone (Fig. 1). The calculated losses of sodium in L. V., J. P. (Fig. 2), and L. S. on 12.5 to 15 mg. of progesterone daily were 68, 46, and 22 meq., respectively. In L. V., the sodium loss on 25 mg. per day was 85 meq., and in J. P. and R. B. the losses during the 50 mg. treatment period were 132 and 138 meq., quantities representative of the usual losses at this dosage. The magnitude of the sodium losses was inevitably impressive and more enduring when 150 to 300 mg. of progesterone per day were given. In J. P. (Fig. 2) the 300 mg. dosage was begun after just 4 days on 150 mg. daily—at a time when the induced natriuresis had begun its characteristic decline. The 8-day loss, however, was 336 meq. In R. B. the loss on 200 mg. daily was 396 meq.; since the 300 mg. course was short and was started during the post-treatment phase of sodium retention, calculations would not be appropriate, but the peak excretion of sodium was higher than at any other time. Entirely similar results were obtained in E. C. (data not shown). Urinary chloride excretion (not shown in Figs. 2 and 3) closely followed that of sodium, but as previously noted[2] the losses were never so great.

As in other experiments in subjects with normal adrenals, immediately following the cessation of treatment with progesterone, urinary sodium excretion dropped abruptly and in the ensuing several days virtually all of the sodium which had been lost was recovered. Following the large doses of progesterone the intensity of the salt retaining impulse was remarkable, the excretion of urinary sodium dropping to levels of only 15 to 25 meq. per day. This contrasts with the treated Addisonians in whom salt loss of the same magnitude was not followed by a sharp retention. It has been suggested[2] that this suppression of urinary sodium may be the result of an increased secretion of adrenocortical salt retaining steroids.

It should be pointed out that in none of the many subjects in whom we have studied the influence of progesterone has there been evidence of preexisting salt and water retention. There is no reason to believe, however, that progesterone might not be saluretic in patients with edema. Leutcher and associates[11] have shown that patients with heart failure, nephrosis, etc., excrete an increased quantity of aldosterone suggesting that this adrenal hormone may play a role in the genesis and/or maintenance of such edema states. If, as has been indicated, the natriuretic influence of progesterone is due to the inhibition of the renal effects of salt retaining corticoids, it is conceivable that progesterone would promote the urinary excretion of salt in such conditions. If so, the post-treatment retention of sodium might be lessened by placing the patient on a low-salt intake.

Although the dosages studied ranged widely from 6.25 to 300 mg. daily, there is every indication that each of these quantities may be presented to the female organism under physiologic conditions. Calculations based on the fact that 10 to 20 per cent of administered progesterone is excreted as pregnanediol[2, 12, 13] indicate that as much as 25 mg. of progesterone may be secreted each day by the corpus luteum of the nonpregnant woman. This deduction is supported by the fact that in L. V. (Fig. 1) 3.3 mg. of pregnanediol per day were excreted during the 25 mg. dosage period and 2.2 mg. per day when 12.5 mg. was given. Both quantities are within the range of pregnanediol excreted during the luteal phase of the menstrual cycle. The 50 mg. dosage probably can be considered as approximating the quantity secreted in early pregnancy, and 200 to 300 mg. per day late pregnancy. J. P. (Fig. 2) excreted 62 mg. of pregnanediol per day when receiving 300 mg. of progesterone. It remains important to demonstrate that endogenous progesterone exerts similar effects, but these data suggest that progesterone might influence protein metabolism and sodium and chloride excretion in the pregnant and the normal menstruating woman.

SUMMARY AND CONCLUSION

1. Progesterone exerts a demonstrable catabolic effect in men and women when administered in dosages which approximate the levels of secretion of the hormone during the luteal phase of the menstrual cycle and early and late pregnancy. Although 6.25 mg. per day had no effect, 12.5 to 15 mg. induced a distinct elevation in urinary nitrogen excretion which averaged about 0.7 Gm. in 3 subjects. Doses of 25 to 50 mg. of progesterone evoked a greater response, the usual nitrogen elevation, averaging 1 to 2 Gm. per day. The intensity of the catabolic effect of progesterone tended to plateau at a dosage of 50 mg. per day. Larger quantities (100 to 300 mg. per day) did not regularly induce greater responses.

2. In one man urinary inorganic phosphorus and potassium dropped significantly during the first 2 or 3 days of progesterone treatment, followed by a return to the base line, and then an overshooting of control levels. A similar slight decline in potassium and inorganic phosphorus had been seen in approximately half of our other studies. Since urinary nitrogen excretion was rising at this time it was suggested that these shifts did not reflect the change in protein metabolism, but some other process.

3. In one mild, controlled diabetic, progesterone did not affect the blood sugar level or induce glycosuria.

4. The intensity and duration of the natriuresis induced by progesterone was clearly related to the dosage. The effect was slight and transient at the low doses, but much greater and more enduring when the dosage reached 150 mg. per day. In all cases the salt loss was recovered during the posttreatment phase of retention.

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SEQUENTIAL ANALYSIS IN THERAPEUTIC RESEARCH

I. Application to Binomial Data and to Measured Data Normally Distributed (One-Sided Alternative)

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THERE is an increasing need for a rapid and reliable method of evaluating pharmacodynamic agents for their potential therapeutic effects. The points for emphasis in the design of the therapeutic experiments are adequate randomization in the double-blind technique, and the accurate estimation of the variation in the variables measured, including the variation due to uncontrollable extraneous factors as well as that due to placebo effects. Usually the design is developed to correspond with the statistical technique by which the results are to be evaluated; thus, the collection of data proceeds until a sizable number of control and test observations have been gathered before a statistical analysis is attempted. Using the sequential method of Wald,[1] however, analysis begins as soon as the data from the control observations are available and continues with each new test observation until a decision is reached. The decision is usually reached and then the testing may be stopped after substantially fewer observations than are required by any other statistical plan now known.[2]

Sequential analysis, originally developed by Wald and associates for quality control in industry, has been applied to biologic and clinical problems at a purely theoretic level[3, 4] and has recently been reported to determine the significance of differences in proportions observed in clinical prophylactic and therapeutic trials[5] (binomial distribution), and to determine the significance of differences in measured variables in various biologic systems in man in terms of the likelihood ratio or the probability ratio tests against a single alternative hypothesis.[6-8] The technique of sequential analysis requires the randomization of the order in which tests are carried out and exploits the tendency of such a distribution to equalize in the long run; i.e., if in tossing an unbiased coin several heads appear without tails, the chance for a tails on the next toss is still "50-50" but it is inevitable that eventually a run of tails will appear so as to ultimately equalize the distribution. Sequential analysis is particularly adaptable to the apraisal of an effect on a biologic system whose intrinsic variability has already been determined, but it requires that this solid base line of control data be available for comparison.

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The first study reported here concerns data that is distributed binomially, in this instance the presence or absence of vomiting following the exhibition of antiemetic agents. The second involves data in which actual measurements were made. The control data, from which the mean and standard deviation were computed, were normally distributed. In this instance the study was designed to measure the day-to-day changes in 24-hour urine volume observed during a control period and during a period of administration of a diuretic agent. Significant change in the measured variable (24-hour urine volume) during treatment occurred in one direction only.

METHODS

The details of the clinical experimental studies herein analyzed as well as the results of their analysis by more standard statistical methods have been reported elsewhere.[9, 10] Briefly, the first study was concerned with the antiemetic effects of 3 identically appearing capsules. The human volunteers on each occasion were given 1 capsule prior to the ingestion of an emetic dose of ipecac. The capsules contained either a placebo, 20 mg. of chlorpromazine (Drug A), or 20 mg. of an experimental antiemetic compound structurally related to chlorpromazine, SKF 4657* (Drug B). The second study involved the measurement of the diuretic effect of two pyrimidinedione compounds, aminometramide† and aminoiso-

TABLE I

WHEN P_0 (%) =	VALUE OF P_1 (%) AT 99% CONFIDENCE INTERVAL WHEN $\bar{n}$ =						
	10	12	16	20	30	60	100
0	(41)	(36)	(28)	(23)	(16)	(8)	(5)
1	43	37	30	25	18	10	7
2	44	38	31	27	20	12	9
3	46	40	33	28	22	14	10
4	47	42	35	30	23	16	12
5	48	43	36	31	25	17	13
6	49	45	37	33	26	18	15
7	51	46	39	34	28	20	16
8	52	47	41	36	29	21	17
9	53	49	42	37	31	23	19
10	54	50	44	39	32	24	20
15	60	56	49	45	38	31	26
20	65	61	55	51	44	37	32
25	69	66	60	56	50	42	38
30	74	70	64	61	55	47	43
35	77	74	69	66	60	52	48
40	81	78	73	70	64	57	53
45	84	81	77	74	69	62	58
50	87	85	81	78	73	67	63
55	90	88	84	82	78	72	68
60	92	91	88	85	82	76	72
65	94	93	91	88	85	80	77
70	96	95	93	91	88	84	81
75	98	97	95	94	92	88	85
80	99	98	97	96	94	91	89
85	(100)	99	99	98	96	95	93
90	(100)	(100)	(100)	99	98	97	96
95	(100)	(100)	(100)	(100)	99	99	99

( ) = Impossible in sequential binomial formulas. Included for interpolation purposes only.

*Smith, Kline, and French, Research Laboratories, Philadelphia, Pa.; 2-chloro-10-[3'-(N-methylpiperzinyl)-propyl]-phenothiazine dimaleate.

†Brand of aminometramide (1-allyl-3-ethyl-6-aminotetrahydropyrimidinedione): Mictine. G. D. Searle & Co., Chicago, Ill.

metradine,* and a placebo administered orally to human subjects with normal cardiovascular renal systems under controlled conditions. Both studies were conducted according to the double-blind technique using a systematized randomization procedure.

The derivation of the sequential graphs was as follows:

Binomial Distribution.—P_0 = unacceptable proportion of therapeutic successes (in this case, the antiemetic responses observed in the placebo control group).

P_1 = acceptable proportion of therapeutic successes (in this case, the antiemetic responses required in the treatment group to be considered significantly better than the placebo control group at an arbitrarily selected 99 per cent confidence interval on the basis of a given number of observations. This value may be obtained from Table I when P_0 is known and the average number of observations to be made has been decided upon. In general, we have found an average of 10 or 12 observations desirable).

As an alternative method, P_1 may be arbitrarily designated by the investigator as the desirable or acceptable proportion of therapeutic successes based upon his past experience and special knowledge in the field of investigation. The formulas for $\tilde{n}\ P_0$ and $\tilde{n}\ P_1$ which follow may then be solved and the average number of observations necessary for a decision will be determined. When P_1 is thus selected and is set too close to P_0 in value, the average number of observations necessary for a decision will become large, but will remain smaller in general than that required by other standard statistical techniques. We have found, however, that by designating the average number of observations as 10 or 12, and then selecting P_1 as described above, one generally obtains a clinically, as well as statistically, acceptable difference between P_0 and P_1. We prefer this technique for general use, as the number of observations needed for decision generally remains small. However, the choice between these two entirely different methods for selecting P_1 rests entirely with the investigators and the clinical and experimental strategies which they wish to employ.

The drawing of a sequential graph requires that there be plotted a lower line to form the margin of one area (in this instance, unacceptable or "reject") and an upper line to form the margin of another area (in this instance, acceptable or "accept"). Between these lines lies the zone of indecision. The lines are partially determined according to the desired values for alpha and beta:

α = the maximum risk or probability of accepting proportions of therapeutic successes equal to P_0 or less (we selected $\alpha = 0.01$).

β = the maximum risk or probability of rejecting proportions of therapeutic successes equal to P_1 or greater (we selected $\beta = 0.01$).

The following notation was used to facilitate the computation of the upper and lower lines[2]:

$$g_1 = \log \frac{P_1}{P_0}$$

$$g_2 = \log \frac{1 - P_0}{1 - P_1}$$

$$a = \log \frac{1 - \beta}{\alpha}$$

(Values for a and b may be obtained from tables in the publication by the Columbia University Statistical Research Group.[2])

$$b = \log \frac{1 - \alpha}{\beta}$$

$$\text{Intercept for lower line} = h_0 = \frac{b}{g_1 + g_2}$$

$$\text{Intercept for upper line} = h_1 = \frac{a}{g_1 + g_2}$$

*Brand of aminoisometradine (1-methallyl-3-methyl-6-aminotetrahydropyrimidinedione): Rolicton. G. D. Searle & Co., Chicago, Ill.

$$\text{Slope} = S = \frac{g_2}{g_1 + g_2}$$

Lower line $= -h_0 + Sn$

Upper line $= h_1 + Sn$

(Solve for $n = 0$, $n = 5$, $n = 10$ to obtain 3 plot points for construction of upper and lower lines).

Theoretical average number of observations necessary to "reject" the agent (lower line) $= \bar{n}\ P_0 = \dfrac{(1 - \alpha)\ h_0 - \alpha h_1}{S - P_0}$.

Theoretical average number of observations necessary to "accept" the agent (upper line) $= n\ P_1 = \dfrac{(1 - \beta)\ h_1 - \beta h_0}{P_1 - S}$.

Measured Values.—The mean and standard deviation of a normally distributed sample (one-sided alternative) is calculated.

n_0 = number of observations in control group.
$\bar{n}_1$ = average number of observations desired in treatment group (arbitrary).
m_0 = mean of control group.
σ = Standard Deviation of control group (degrees of freedom $= n_0 - 1$).
m_1 = mean required in treatment group to be considered significantly different from the control group at a desired probability (we used $P = 0.01$), when $\bar{n}_1$ is set at a desired number (we used $\bar{n}_1 = 6$).

m_1 may be found by solving the following equation where all other values are known or arbitrarily selected, and by letting $\bar{n}_1 = \bar{n}\ m_1 = 6$.

$$\bar{n}\ m_1 = \frac{(1 - \beta)\ a - \beta b}{(m_1 - m_0)^2/2\sigma^2}$$

Note: As an alternative, one may set m_1 arbitrarily at any desired level for the mean required in the treatment group to be considered clinically significant as compared to the known m_0 value, and based upon the investigator's past experience and special knowledge in the field of investigation. The formulas for $\bar{n}\ m_1$ and $\bar{n}\ m_0$ may then be solved and the average number of observations necessary to reach a decision thus determined. However, if m_1 is set too close in value to m_0, the average number of observations necessary for decision will become large, but will remain smaller than that required by any other known statistical plan. In general, we have found that designating $\bar{n}_1$ $(\bar{n}\ m_1) = 6$ leads to an acceptable clinical as well as statistical difference between m_0 and m_1, and we prefer this method for general use, as the number of observations needed for decision generally remains small. The choice between these two methods for selecting m_1 again depends upon the clinical and experimental strategies desired by the investigators.

α = arbitrarily selected security risk of accepting m_1 when m_0 is true (we used $\alpha = 0.01$).

β = arbitrarily selected security risk of rejecting m_1 when m_1 is true (we used $\beta = 0.01$).

$a = \log_e \dfrac{1 - \beta}{\alpha}$ (Values for a and b may be obtained from tables in the publication by the Columbia University Statistical Research Group.[2])

$b = \log_e \dfrac{1 - \alpha}{\beta}$

$h_1 = \dfrac{a\sigma^2}{m_1 - m_0}$ = Upper intercept

$h_0 = \dfrac{b\sigma^2}{m_1 - m_0}$ = Lower intercept

$S = \dfrac{m_0 + m_1}{2}$ = Slope

Lower line $= -h_0 + Sn$

Upper line $= h_1 + Sn$

(Solve for $n = 0$, $n = 5$, $n = 10$, to obtain 3 plot points for construction of upper and lower lines.)

Note: The Slope (S) was subtracted from each result here. This step is necessary only if one wishes to keep the upper and lower lines of the sequential graph horizontal and parallel to the abscissa and at right angles to the ordinate.

Theoretical average number of observations

necessary to reach a decision at m_0 (lower line) $= \bar{n}\ m_0 = \dfrac{(1-\alpha)\ b - \alpha}{(m_1 - m_0)^2/2\sigma^2}$.

Theoretic average number of observations

necessary to reach a decision at m_1 (upper line) $= \bar{n}\ m_1 = \dfrac{(1-\beta)\ a - \beta b}{(m_1 - m_0)^2/2\sigma^2}$.

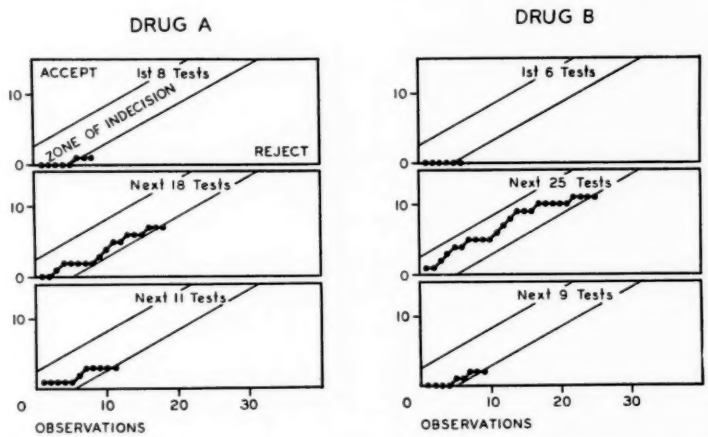

Fig. 1.—The individual results of tests with Drug A and Drug B measuring their effectiveness in producing "protection" against ipecac-induced vomiting compared to the placebo control, plotted sequentially until an answer is obtained on 3 successive graphs. The lack of complete "protection" against vomiting is plotted directly one square to the right for each observation and its presence is plotted one square up as well as one square to the right. Thus the ordinate represents the number of "protection" responses and the abscissa the number of observations. Both Drug A and Drug B were repeatedly rejected as not having an effect significantly greater than the placebo control.

RESULTS

1. *Binomial Data.*—In the experiments concerning the attempted prevention of ipecac-induced vomiting by premedication with various antiemetic agents, we found on the basis of 83 placebo medication tests an incidence of 34 per cent protection against vomiting (the incidence of no vomiting in the nonpremedicated "control" tests was 15 per cent). The data from the placebo observations were binomially distributed. They were thus suitable for use as the control group for the construction of the sequential graph as follows:

$P_0 = 0.35$ for "protection" against vomiting (the closest to 34 per cent in Table I). Thus, $P_1 = 0.74$ on the basis of $\bar{n}_1 = 12$, the arbitrarily selected average number of tests desired for each agent to be tested. We selected 0.01 as the alpha and beta risks (a generally conceded safe level for clinical interpretation) and solving the sequential formulas for a binomial distribution, $S = 0.5562$, $h_0 = 2.6757$ and $h_1 = 2.6757$. From these the sequential graph was constructed (Fig. 1) and the results of tests with Drug A and Drug B were plotted in sequential fashion as they were performed. There were a total of 49 tests with Drug A and 52 tests with Drug B. Drug A gave an over-all incidence of 43 per cent "protection" and Drug B 37 per cent "protection" against

vomiting. Drug A is rejected on the sequential graph on the first 8 tests and also by plotting separately the next 18 tests and the next 12 tests. Drug B is rejected on the first 6 tests and also by plotting separately the next 26 tests and the next 9 tests (theoretic average numbers of tests required here are 14 for acceptance and 13 for rejection). Thus, all of the conclusions reached by sequential analysis for both Drug A and Drug B, concerning their effectiveness against vomiting, are that neither agent is significantly better than the placebo. Ordinarily, one would stop the experiment with a test agent after the first decision is reached. Here, analysis of both drugs was continued using further observations until 3 decisions with each drug were reached, primarily to indicate the consistency of decision by this technique. Analysis of this data by more standard statistical techniques has been reported previously.[9]

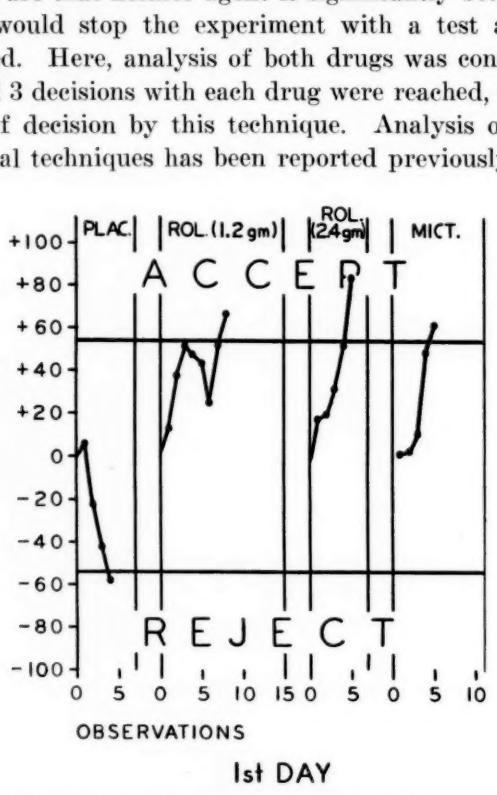

Fig. 2.—The results of the changes in urine volume observed during the first day of the administration of a placebo, of 1.2 and 2.4 Gm. of Rolicton and of 1.2 Gm. of Mictine compared to the control plotted sequentially and in summation fashion until an answer is obtained for each drug treatment group. The abscissa represents n (the number of observations) and the ordinate the Σ (X-S) (the sum of each change in 24-hour urine volume minus the slope observed on the first treatment day). Mictine and Rolicton, in both the small and large dose, are accepted on the first day as inducing an increase in urine volume significantly greater than that observed during the control period. The placebo is rejected on the first day as not inducing a significant change in urine volume compared to the control.

2. *Measured Data.*—In the experiments in which the diuretic activity of various compounds was measured in healthy subjects the urine volume measurements observed in the control observations were normally distributed. There were 23 subjects tested. The 24-hour urine volume was expressed as per cent of intake (output/intake × 100); the mean for each subject for the 3 control days was calculated and the daily variation from this mean was recorded not only for the 3 control days but also for each of the 3 treatment days that followed. Only the first treatment day is reported here. The standard deviation

of the control observations was 14.1. Solving the formula given previously under methods we find $m_1 = 17.3$ using $\bar{n}_1 = 6$. Alpha and beta are again selected as 0.01 and solution of the appropriate formulas yield $h_0 = 52.84$, $h_1 = 52.84$, $S = 8.6$. Fig. 2 presents the results plotted on the sequential graph thus constructed, from the first treatment day for each drug tested, including the placebo. The Slope (S) was subtracted from each individual observation as was done in the derivation of this sequential graph (see methods). The placebo was rejected on the sequential graph as it failed to induce a change in the 24-hour urine volume which differed significantly from the control observations. Aminoisometradine (Rolicton) was accepted on the first treatment day as having diuretic properties in both the dose of 1.2 Gm. and 2.4 Gm. per day, and aminometramide (Mictine) was accepted on the first treatment day at a dose of 1.2 Gm. per day. The number of tests required to make the above decisions was 4 for the placebo, 8 for the 1.2 Gm. dose of aminoisometradine, 5 for the 2.4 Gm. dose of aminoisometradine, and 5 for the 1.2 Gm. dose of aminometramide. The individual observations from this experiment have been reported previously in tabular form, together with analysis of the data by more standard statistical techniques.[10]

DISCUSSION

The technique of sequential analysis has been found convenient and reliable when applied to clinical therapeutic experiments and yields a satisfactorily accurate decision regarding the efficacy of the agent tested with fewer observations than are required by more standard statistical techniques currently in use. By plotting the result of each individual observation as it is made, it permits one to stop an experiment as soon as a decision is reached. It is readily applicable to clinical data binomially distributed and to measured data normally distributed.

Several deviations from the usual methods of applying sequential analysis to clinical data are described. First, in dealing with a binomial distribution, instead of arbitrarily selecting P_1 when P_0 is known, and then calculating the average number of observations ($\bar{n}$ P_1) needed to establish statistical difference, we have chosen to arbitrarily fix $\bar{n}_1$ at a desirable number and then to determine P_1 (from Table I). That the differences between P_0 and P_1 will also be of clinical significance is practically insured if $\bar{n}_1$ is kept at 10 or 12. Second, in dealing with measured data the usual plans have most often selected m_1 as 2σ from m_0, or at another purely arbitrary distance from the known m_0 and then calculated the average number of observations ($\bar{n}$ m_1) needed to demonstrate a significant difference. We have chosen to arbitrarily fix $\bar{n}_1$ (or $\bar{n}$ m_1) at a desirable number and then calculate m_1 by the method given. In our experience, when m_1 is arrived at in this way it has always been acceptable as being significantly different clinically from m_0. Should it not be, however, one may widen the gap between m_0 and m_1 arbitrarily by raising the value of m_1 and then recalculating $\bar{n}$ m_1; or, should the difference seem clinically too great, the gap may be arbitrarily narrowed and $\bar{n}$ m_1 recalculated. It is doubtful that either of these maneuvers shall prove necessary very often. Finally, in

the construction of the one-sided alternative sequential graph dealing with measured values we have chosen to routinely subtract the Slope (S) from each calculated value for the upper and lower lines and from each observation value to be plotted. This changes the appearance of the usual sequential graph so that it no longer has slanting upper and lower lines, but rather horizontal upper and lower lines which remain parallel to the abscissa and at right angles to the ordinate of the graph. This in no way alters the decisions reached, or their significance.

SUMMARY

A method is presented whereby the statistical technique of sequential analysis has been applied to clinical data binomially distributed as well as to measured data normally distributed. This technique permits the plotting of each individual test observation as it is made, and permits stopping the experiment when a decision is reached. Further, it generally requires fewer observations than other more standard statistical techniques currently in use. It should become a valuable aid for the evaluation of therapeutic agents.

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SEQUENTIAL ANALYSIS IN THERAPEUTIC RESEARCH

II. Application to Measured Data Normally Distributed (Two-Sided Alternative)

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EARLIER adaptations of sequential analysis to clinical data have allowed for a bidirectional alternative in data binomially distributed and for the detection of a significant change in measured variables in one direction, or the detection of a significant change in measured variables in either direction without indicating the direction of the change.[1-9] The present report concerns the adaptation of sequential analysis to the study of the effects on various biologic systems in man of pharmacodynamic agents which may produce significant changes in measured variables in one direction or the other from the control observations, in order to indicate not only significant change but also the direction of that change (the two-sided alternative).

MATERIAL AND METHODS

Data from three separate clinical studies designed to measure the pharmacodynamic effects of certain therapeutic agents are described.

The first study involved the effects of reserpine† and an anticholinergic gastric inhibitor (AP 396)‡ on gastric acidity. Patients from the general medical wards served as subjects and comprised primarily those recently recovered from an acute illness without known gastrointestinal tract disease. A few patients with past histories suggesting chronic duodenal ulcer and minor upper gastrointestinal tract symptoms were included. Following a 12-hour fast, 3 samples of gastric juice were collected at 15-to 30-minute intervals during a ½- to 1-hour control period. Attempts were made during the collection of these and all subsequent samples to empty the stomach as completely as possible. After the control period the agent to be tested, dissolved in 30 ml. of water, was administered through the Levin tube. The tube was clamped for 1 to 1½ hours and then sampling was resumed at 30-minute intervals for 3 hours (7 postmedication samples). For completion of the study, 42 subjects were given a placebo, 19 a single dose of 2.5 mg. of reserpine, and 22 a single dose of 40 mg. of AP 396. The mean free acid, expressed as meq. HCl per liter, as determined with Toepfer's reagent and 0.1 N NaOH (accuracy ± 5 meq./L.), was calculated for each subject for the premedication (control) and for the postmedication period, as well as the difference between the mean free acidity of the control and the postmedication periods (the Δ mean free HCl). The mean free acidity value for each of the 3 medication groups was also calculated for the control and the postmedication periods (Table I).

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†Brand of crystalline alkaloid reserpine: Rau-sed. E. R. Squibb & Sons, New York, N. Y.

‡Experimental antispasmodic and antigastric-secretory drug (diethylaminoethyl beta-hydroxy-x-phenylcyclohexane-acetate methobromide); Abbott Laboratories, North Chicago, Ill.

The second study was designed to measure the effects of various compounds upon the level of the blood sugar. The venous blood glucose (modified Somogyi method) (accuracy ± 5 mg. per cent) was determined 2 hours after a standard oral glucose load (1.75 Gm. per kilogram of ideal body weight). On various test days the oral glucose load was preceded 8½ and 2 hours before by the ingestion of 50 mg. cortisone, 10 mg. prednisone, and 10 mg. prednisolone by 21 subjects; on 1 other occasion it was preceded by 2 mg. 9-alpha-fluorohydrocortisone by 16 of the same subjects; and on still another occasion by 2.0 Gm. carbutamide by 12 of the same subjects. Each of the 21 subjects also had a control test (no medication prior to the ingestion of glucose).

TABLE I

SUBJECT NO.	FREE HCl MEQ./L. MEAN CONTROL (3 OBS.)	FREE HCl MEQ./L. MEAN POSTMED. (7 OBS.)	Δ MEAN FREE HCl (MEAN POSTMED. MINUS MEAN CONTROL)
Placebo 1	40	36	− 4
2	52	56	+ 4
3	55	58	+ 3
4	73	70	− 3
5	34	42	+ 8
6	17	11	− 6
7	48	42	− 6
8	51	57	+ 6
9	51	39	−12
10	0	23	+23
11	60	66	+ 6
12	8	11	+ 3
13	2	16	+14
14	0	1	+ 1
15	5	0	− 5
16	10	39	+29
17	25	26	+ 1
18	42	42	0
19	42	53	+11
20	7	28	+21
21	12	11	− 1
22	10	34	+24
23	45	49	+ 4
24	45	51	+ 6
25	15	46	+31
26	77	36	−41
27	28	51	+23
28	18	36	+18
29	75	76	+ 1
30	13	32	+19
31	27	63	+36
32	53	46	− 7
33	23	51	+28
34	45	51	+ 6
35	63	79	+16
36	27	9	−18
37	53	56	+ 3
38	28	48	+20
39	10	21	+11
40	40	29	−11
41	22	54	+32
42	35	69	+34
Placebo group mean	33.0	41.0	+7.8 (Placebo control)
Reserpine 1	15	60	+45
2	17	55	+38
3	13	39	+26
4	15	45	+30
5	11	79	+68
6	42	72	+30
7	42	76	+34
8	50	103	+53
9	0	33	+33
10	50	69	+19
11	25	67	+42
12	35	91	+56
13	52	54	+ 2
14	27	39	+12
15	68	92	+24
16	18	70	+52
17	18	60	+42
18	50	41	− 9
19	0	14	+14
Reserpine group mean	28.8	60.7	+32.0
AP 396 1	52	50	− 2
2	40	37	− 3
3	65	3	−62
4	65	58	− 7
5	23	3	−20
6	33	14	−19
7	38	24	−14
8	30	21	− 9
9	22	0	−22
10	23	3	−20
11	27	0	−27
12	18	3	−15
13	38	23	−15
14	33	14	−19
15	30	3	−27
16	13	3	−10
17	22	6	−16
18	20	13	− 7
19	15	16	+ 1
20	33	5	−28
21	38	1	−37
22	28	22	− 6
AP 396 group mean	32.3	14.5	−17.5

Note: The difference between 41–33 = 8.0 and 7.8, 60.7–28.8 = 31.9 and 32.0, and 14.5–32.3 = −17.8 and −17.5 are due to "rounding off" of figures in calculations.

The third study included observations on 300 subjects. There were 10 different eyedrops tested. For each medication there were 10 white subjects tested with blue eyes, 10 white subjects with brown eyes, and 10 Negro subjects with dark eyes. The nature of the drops were unknown to both the investigators and the subjects. The medications evaluated were normal saline (Placebo 1), 4 per cent boric acid (Placebo 2), 10 per cent Neosynephrine, 3 per cent ephedrine, 2 per cent pilocarpine, 0.2 per cent scopolamine, 1 per cent Cyclogyl, 1 per cent atropine, 4 per cent homatropine, and 1 per cent Paredrine. The pupil size was measured with a millimeter ruler at a standard distance from the eye, 3 drops of the medication to be tested were instilled in the eye, and the pupil size was again measured 40 minutes later, using the same technique. Identical measurements were also carried out on the other eye, which received no drops. The results of the 60 tests performed on the other eyes of those subjects receiving placebo drops were later utilized as the control observations. In all, 303 tests were eventually completed in this study, an additional 3 tests being performed in Negroes with dark eyes using 2 per cent pilocarpine.

More detailed information concerning the design and individual observations from the second and third studies are presented in separate reports.[10, 11]

The sequential graphs presented here were all derived from the same basic formulas, the application of which has been variously described[1-8] and recently further modified.[9]

The calculations used for construction of these sequential graphs were identical with those modifications described for normally distributed measured variables[9] except here the Slope (S) was not subtracted from each value, thus allowing the lines of the graph to remain slanting. A sequential graph so constructed will permit measurement of a significant change in one direction, as a significant increase over and above the established control values. However, the plot lines of a drug inducing a significant decrease and of a drug inducing no significant change from the control observations will both cross the lower line of the graph and thus be rejected, but will not be differentiated one from the other. This is inherent in the one-sided alternative plan. In order to further modify the graph to make this latter differentiation, one may expand the analysis as follows:

The value $m_1 - m_0$ is known from the first calculation, as is the value of m_0 (9). One may then solve the following equation for m_2 (where m_2 is the mean required in the treatment group for indicating a significant decrease in the values of the measured variables from those observed in the control group with a probability of $P = 0.01$ when $\bar{n}_2 = 6$: $m_0 - m_2 = m_1 - m_0$.

Allow α and β to remain the same; a and b will thus remain the same and h_1 and h_0 will remain the same.

Note: As an alternative method, the value of m_2 may be arbitrarily assigned, based upon the past experience of the investigator and his special knowledge in the field of study. The average number of observations may then be calculated from the formula previously given[9] substituting $\bar{n}\ m_2$ for $\bar{n}\ m_1$ and $m_0 - m_2$ for $m_1 - m_0$. The problem of increasing the average number of observations needed for a decision to an undesirably large size exists if one assigns the value for m_2 too close to that of m_0. We thus prefer the method given above for general use (allowing $\bar{n}_2$ [$\bar{n}\ m_2$] to arbitrarily be 6), as it usually leads to satisfactory clinical as well as statistical significance and generally permits the average number of observations to remain small.

Then solve for a new slope:

$$S' = \frac{m_0 + m_2}{2}$$ (where S′ is the slope of the lower portion of graph).

Lower line $= -h_0 + S'\ n$

Upper line $= h_1 + S'\ n$

Solve again for $n = 0$, $n = 5$, $n = 10$, to obtain 3 plot points for construction of upper and lower lines of lower portion of graph.

Note: The upper line of the lower portion of the graph will now intersect the lower line of the upper portion of the graph. The lines are arbitrarily not drawn to the left of the area of intersection, although they may be so drawn if desired.[5]

The resulting graph will now outline the zone of indecision and delineate "above," an area indicating a significant increase over the control values; "in the middle," an area indicating no significant change from the control values; and "below," an area indicating a significant decrease from the control values.

The abscissa of the graph constructed represents n_1 (number of observations of the test group) and the ordinate represents the ΣX (the sum of the measured variables in the test group). The individual measured variable of each test observation is plotted in summation fashion, one after the other for each test group as they are sequentially and randomly obtained, until a decision is reached. A decision is reached when the plot line of a test group crosses out from the zone of indecision into one of the three areas of decision.

The data utilized as the control in each of the three studies herein described were normally distributed.

RESULTS

In the study of gastric acidity, none of the mean "control" (premedication) values for free acidity (33.0, 28.8, 32.3) differed significantly from each other in any of the 3 medication groups (Table I). In view of the fact that the observations obtained after medication provided a two-sided alternative (a significant increase or decrease in gastric acidity as compared to the control) they appeared suitable for the attempt at interpretation by sequential analysis, modified as described above. The placebo postmedication period demonstrated a slight increase in mean free HCl of borderline significance when compared to its premedication control by *t*-test (P less than 0.1 and greater than 0.05) (Fig. 2). The placebo Δ mean free HCl values were thus used as the control data upon which to base the sequential analysis, since a significant difference from these values was the object of clinical search. Table II summarizes the basic data utilized for construction of all the sequential graphs presented in this paper.

TABLE II

BASIC DATA UTILIZED IN CONSTRUCTION OF THE SEQUENTIAL GRAPHS		GASTRIC FREE HCl STUDY	BLOOD GLUCOSE STUDY	EYEDROP STUDY
Control group	=	Data from placebo observations (Δ mean free HCl)	Data from no premedication tests	Data from observations on the other eye of those subjects receiving placebo drops
n_0 (number of control observations)	=	42	21	60
m_0 (control mean)	=	+ 7.8	78.29	+0.08
Sigma (Standard Deviation)	=	15.7	14.03	0.91
m_1	=	+26.96	+95.41	+1.19
m_2	=	−11.36	+61.17	−1.03
α	=	0.01	0.01	0.01
β	=	0.01	0.01	0.01
Intercepts:				
h_0	=	59.18	52.89	3.44
h_1	=	59.18	52.89	3.44
Slope (upper portion)	=	+17.38	+86.85	+0.63
Slope' (lower portion)	=	− 1.78	+68.73	−0.48

When the individual Δ mean free acidity values (mean postmedication minus mean control) are plotted from Table I for each subject in sequential fashion for each drug treatment group as illustrated in Fig. 1, reserpine is

accepted as a significant stimulator agent on the first 4 observations, and AP 396 is accepted as a significant inhibitor agent on the first 7 observations.

The experiment was continued to gain larger numbers of observations (83 total) in the various medication groups and these results were analyzed

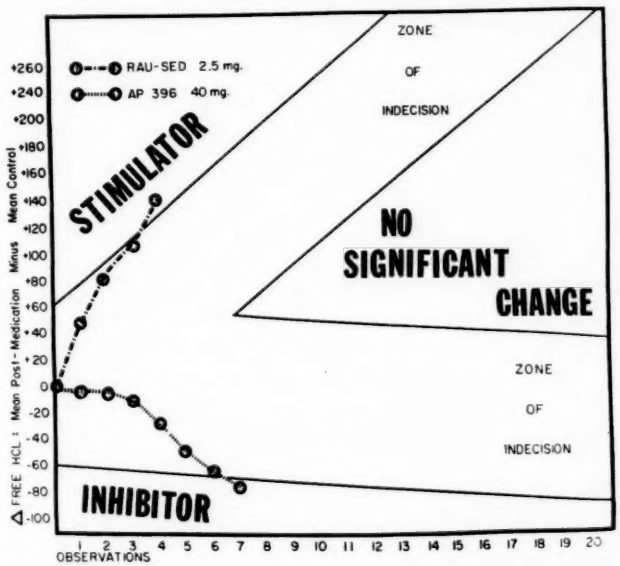

Fig. 1.—The sequential graph illustrating that reserpine was found to be a significant stimulator of gastric acidity on 4 observations and AP 396 a significant inhibitor of gastric acidity on 7 observations utilizing the Δ mean free HCl observations from the placebo group as the control. The abscissa represents n (the number of observations) and the ordinate the ΣX (the sum of the Δ mean free HCl of each observation). These are plotted in sequential and summation fashion for each subject in each drug test group until an answer is obtained.

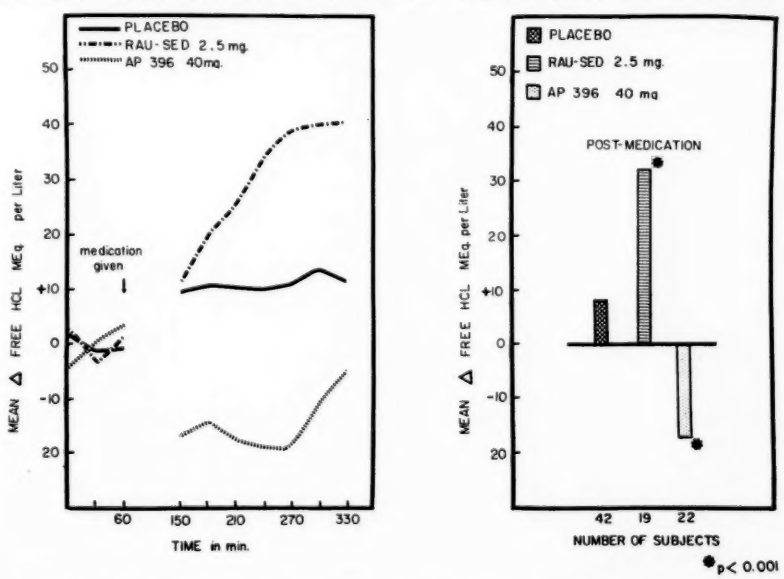

Fig. 2.—*Left*, The mean Δ free acidity, after all 83 tests were completed, (mean of postmedication observations minus mean control) for each time interval for each of the 3 drug treatment groups in the control (premedication) and postmedication period. *Right*, The mean Δ free acidity after all tests were completed (mean of postmedication observations minus mean of control observations) for each of the 3 drug treatment groups. The P values are from *t*-tests using the mean Δ free HCl of the placebo group as the control.

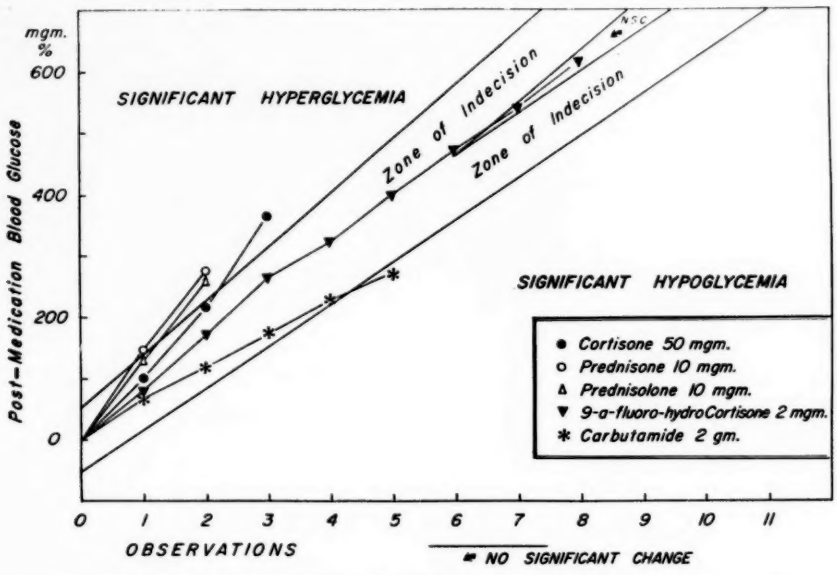

Fig. 3.—The sequential graph designed to measure significant hyperglycemia, no significant change or significant hypoglycemia compared to the control. Cortisone, prednisone, and prednisolone were found to induce significant hyperglycemia on 3, 2, and 2 observations, respectively, 9-alpha-fluorohydrocortisone to have no significant effect on 7 observations, and carbutamide to induce significant hypoglycemia on 5 observations. The abscissa represents n (the number of observations) and the ordinate the Σ X (the sum of the blood glucose values observed in the drug test groups). The results are plotted in sequential and summation fashion for each drug test group until an answer is obtained.

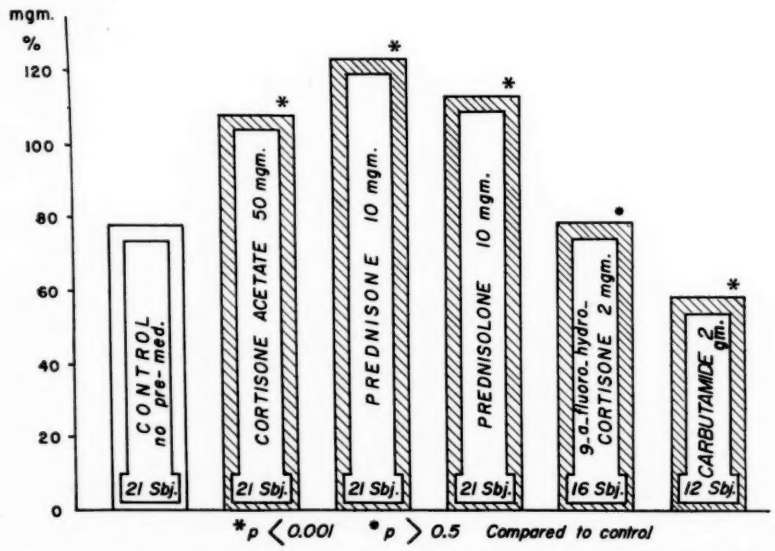

Fig 4.—The group mean venous blood glucose, after completion of the 112 tests, 2 hours after ingestion of an oral glucose load for the control, and repeated after premedication with 50 mg. cortisone acetate, 10 mg. prednisone, 10 mg. prednisolone, 2 mg. 9-alpha-fluorohydrocortisone, and 2.0 Gm. carbutamide. All medications led to a significant alteration in blood glucose levels compared to the control except 9-alpha-fluorohydrocortisone, which induced no significant change. The P values are from *t*-tests comparing each medication group to the control.

by a standard statistical technique (*t*-test). The rise in the level of the mean free HCl observed in the reserpine postmedication period (+32.0 meq./L.) was significantly different from the placebo control (+7.8 meq./L.) (P less than 0.001) and the fall observed in the AP 396 postmedication period (–17.5 meq./L.) was also significant when compared to the placebo control (P less than 0.001). (Fig. 2.)

For the venous blood glucose studies, the sequential graph was constructed from the absolute values of the control data (no premedication) according to the technique previously described under Methods. The analysis utilizing this graph is presented in Fig. 3. The individual value for each subject's 2-hour blood glucose determination for each drug treatment group was plotted in sequential and summation fashion until an answer was obtained. When the answer was evident after plotting the first test, a second test was plotted to insure that the decision remained unchanged. This technique permitted the conclusion that, compared to the control, cortisone induced a significant rise in blood glucose (only 3 tests required for decision), as did prednisone and prednisolone (2 tests with each required for decision), and that 9-alpha-fluorohydrocortisone induced no significant change in blood glucose from the control observations (7 tests required for decision). The conclusion that carbutamide led to a significant fall in blood glucose was reached in 5 tests.

The experiments were continued until there were larger numbers in the various medication groups (112 tests total), and the results were then analyzed by the *t*-test. Fig. 4 summarizes the results of these further studies. The P values for the *t*-tests performed permit the conclusion that cortisone, prednisone, and prednisolone, in the dosages employed, significantly elevate the blood glucose 2 hours after a standard oral glucose load compared to the control (P less than 0.001 in each instance), and that 2 mg. of 9-alpha-fluorohydrocortisone induces no significant change in the blood glucose value compared to the control (P greater than 0.5). Carbutamide in a dose of 2.0 Gm. leads to a significant fall in the blood glucose level (P less than 0.001).

The sequential graph for analysis of the eyedrop tests, also constructed according to the technique described above, was based on the variation observed in the 60 control observations carried out in the other eyes of those subjects receiving placebo drops. Fig. 5 (upper half) illustrates that the conclusion that scopolamine, atropine, Paredrine, homatropine, and Cyclogyl induced significant mydriasis in white blue eyes, white brown eyes, and Negro dark eyes was reached in each instance with only 2 to 5 individual tests plotted in sequential and summation fashion for each drug group. Fig. 5 (lower half) illustrates that the conclusion that neither placebo induced a significant change in pupil size compared to the control observations in white blue eyes, white brown eyes, or Negro dark eyes was reached with only 7 to 10 tests plotted in similar fashion for each group tested. Further, the conclusion that Neosynephrine did induce significant mydriasis in all 3 eye-type groups is reached in 4, 2, and 5 tests, respectively. Of particular interest here is the conclusion that ephedrine induces significant mydriasis in white blue eyes was reached in 3 tests, and in white brown eyes in 7 tests, and the conclusion that it does not induce significant mydriasis in Negro dark eyes was reached in 9

tests. The conclusion that pilocarpine induces significant myosis in white blue eyes was reached in 4 tests, in white brown eyes in 4 tests, but in Negro dark eyes 11 tests were required for a decision.

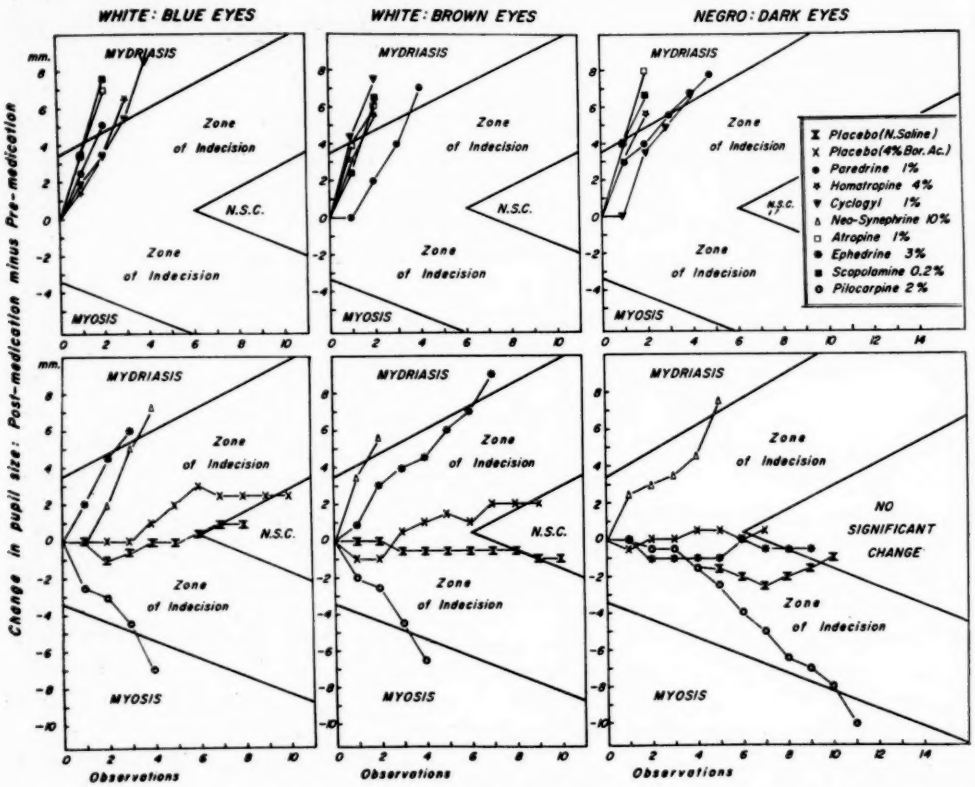

Fig. 5.—*Upper half*, The sequential graph constructed in order to measure significant mydriasis, no significant change or significant myosis compared to the control observations. Cyclogyl, Paredrine, scopolamine, atropine, and homatropine are found to induce significant mydriasis in all eye types with no more than 5 tests with any drug in any eye-type group being required in order to reach this decision. *Lower half*, The same sequential graph reproduced and illustrating that the decision that neither placebo induced a change significantly different from the control in all eye-type groups was reached on from 7 to 10 tests on every occasion, that the decision that Neosynephrine does induce significant mydriasis in all eye-type groups was reached on 4, 2, and 5 tests, and that pilocarpine does induce significant myosis in all eye-type groups was reached on 4, 4, and 11 tests. Ephedrine was found to induce significant mydriasis in white blue eyes on 3 tests, in white brown eyes on 7 tests, and was found to be totally ineffective in Negro dark eyes on 9 tests. The abscissa represents n (the number of observations) and the ordinate the ΣX (the sum of the changes in pupil size observed in each group after medication). The results are plotted in sequential and summation fashion for each drug test group in each eye-type group until an answer is obtained.

The experiments were continued until larger numbers were gathered in the various medication groups (303 total) and the results were then analyzed by the *t*-test. Fig. 6 illustrates these results. The mean change in pupil size for each drug treatment group is plotted in the upper portion of the graph for white blue eyes, white brown eyes, and Negro dark eyes, as well as for the control observations individually by eye type and also combined, and for the placebo observations individually by eye type. No significant differences between the results of the placebo tests and those of the control observations were demonsrated (P less than 0.5 and greater than 0.1). Scopolamine, atropine, and homatropine, as well as Neosynephrine, Paredrine, and Cyclogyl, induced significant dilatation of the pupil in all 3 eye-type groups (P less than

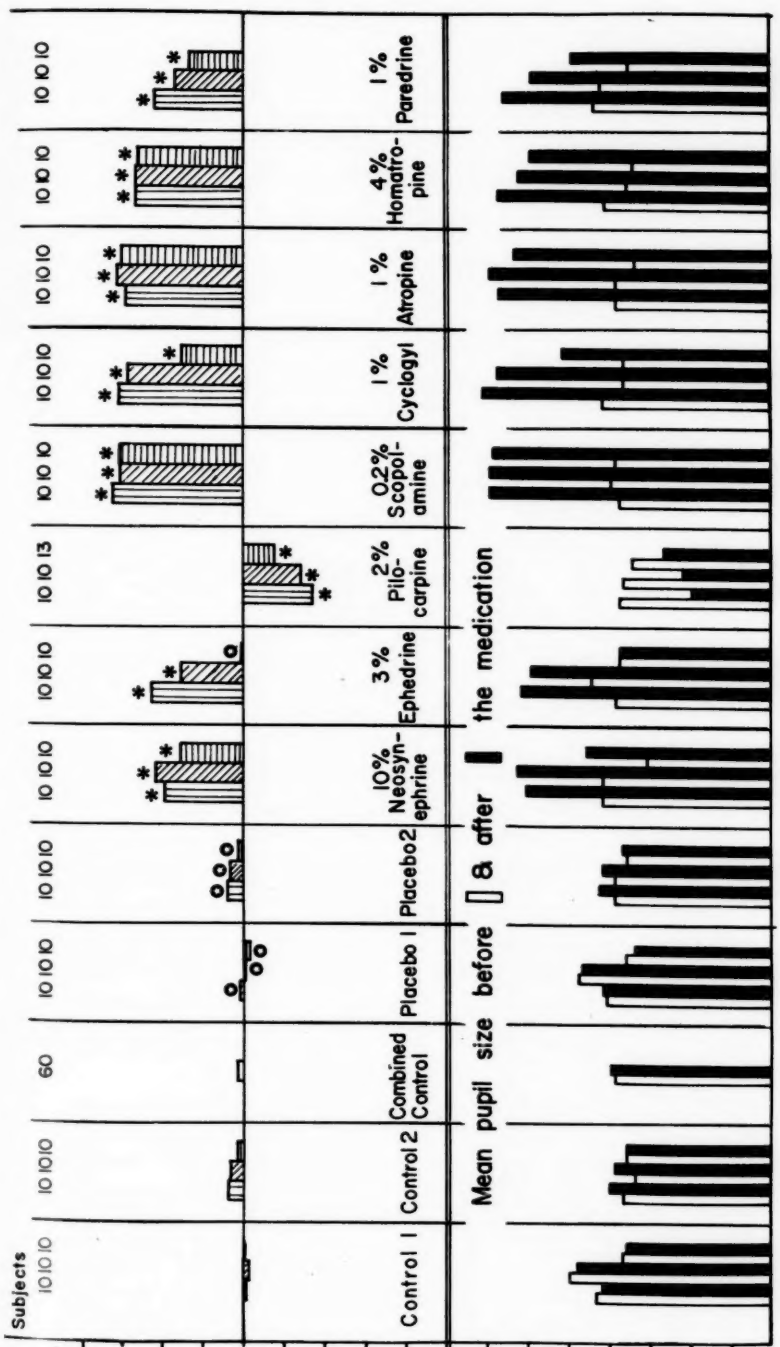

Fig. 6.—The mean change in mean pupil size (in millimeters) following medication after 303 tests were completed; for the control groups, changes are expressed individually by eye type and also for the results of all 60 control tests combined, for the placebo trials expressed individually by eye type, and for each test agent expressed individually by eye type. None of the results of the placebo tests differed significantly from the control observations. Neosynephrine, Cyclogyl, Paredrine, scopolamine, atropine, and homatropine induced significant mydriasis in all eye types, although the first 3 were slightly less potent in Negroes. Pilocarpine induced significant myosis in all eye types but was considerably less potent in Negroes. Ephedrine induced significant mydriasis in white subjects with blue eyes and brown eyes, but failed completely in Negro subjects with dark eyes. The P values are from *t*-tests using the 60 observations on the untreated eyes of the placebo groups as the control. The lower portion of the graph indicates the actual mean pupil size in millimeters for each eye-type group in each medication group before and after medication.

0.001 in each instance), though Cyclogyl and, to a lesser extent, Neosynephrine and Paredrine were somewhat less effective in the Negro dark eyes. Ephedrine induced significant mydriasis in white blue eyes and brown eyes (P less than 0.001 in both instances) but failed to significantly alter pupil size in Negro dark eyes (P less than 0.5 and greater than 0.1). Pilocarpine induced significant myosis in all 3 eye-type groups (P less than 0.001 in each instance) but was considerably less potent in Negro dark eyes (it required 11 observations to reach a decision here on the sequential graph).

SUMMARY AND CONCLUSIONS

Three studies involving the measurement of the pharmacodynamic effects of various agents on human subjects are presented. A method is described adapting the technique of sequential analysis to the interpretation of the data obtained in these studies, all of which included the possibility of a significant increase and a significant decrease after treatment from the measured variables in the control group. The data from the control group in each study were normally distributed, a criterion which must be met before sequential analysis as herein described can be utilized.

Eighteen different pharmacodynamic agents acting on 3 widely differing bodily processes were tested in 498 separate trials appropriately controlled by a placebo double-blind technique. In each instance sufficient observations were made to allow further analysis of the data by a standard statistical technique. The sequential graphs, however, yielded valid inferences after a relatively small number of observations, which were in each instance consistent with the inferences later drawn from the more commonly used and reliable *t*-test.

The sequential plan described should be applicable in many areas of clinical investigation where measured variables are involved and a bidirectional or two-sided alternative exists. Sequential analysis should become an important and useful tool in therapeutic research.

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THE EFFECT OF GRADIENT-TYPE HYDROGEN-ION CONCENTRATION PLATES ON ANTIBIOTIC SENSITIVITY TESTS

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THE method of determining bacterial sensitivity to antibiotics by the use of dried, impregnated filter paper disks on solid media, is standard procedure in many clinical laboratories. However, it is well recognized that a number of variable factors may influence the results of this type of test. These include (1) variations in the nature and composition of the medium, (2) the depth of the agar, (3) the hydrogen-ion concentration of the medium, (4) the size and age of the bacterial inoculum, (5) the duration of incubation, (6) the amount of surface moisture on the agar, and (7) the concentration of antibiotic in the filter paper disk. Each of these factors independently or in combination may affect the final result and close attention to technique is required if accurate and reproducible results are to be obtained.

Of these variable factors the influence of changes in the pH of the medium is of considerable importance. The present study was undertaken in view of the observation of conflicting results obtained with different batches of agar. These were found to be due to differences in the final pH of the media. The method employed consists of a modification of the gradient-type agar plate described by Szybalski.[1] The plates were prepared according to the method of Sacks[2]; the gradient pH was produced by the incorporation of different buffer solutions.

MATERIALS AND METHODS

Antibiotics.—Stock solutions of crystalline penicillin G (1,000 units per milliliter), chlortetracycline, oxytetracycline, tetracycline, streptomycin sulphate and chloramphenicol (each 1,000 μg per milliliter) were prepared in sterile distilled water. Solutions of erythromycin and spiramycin were prepared by dissolving 50 mg. of the pure substance in 1.0 ml. of ethanol and then diluting with sterile distilled water to a final concentration of 1,000 μg per milliliter.

The stock solutions were stored in the Deepfreeze and suitable dilutions were prepared in sterile distilled water as required. The potency of the stock solutions remained constant during the period of the investigation.

Gradient pH Plates.—Sterile molar solutions of K_2HPO_4 and KH_2PO_4 were prepared. The former was added to 10 ml. of melted and cooled nutrient agar (1:10 v/v) which was then poured into a sterile Petri plate. The agar was allowed to solidify with the plate held in an inclined position so that a wedge-shaped area of agar was produced. This was most conveniently done by resting the bottom of the plate, near its center, on a piece of glass tubing. A second aliquot of melted agar containing KH_2PO_4 (1:10 v/v) was then poured on top of the first layer with the plate held in a horizontal position. Diffusion of the buffer solutions took place rapidly between the two layers and the plates were ready for use in 2 to 3

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hours. The range of pH across the plates was determined by laying strips of filter paper containing indicator solutions, across the surface. The method gives a satisfactory and uniform change of pH from approximately 5.4 on one side to 8.0 on the other.

All plates were used on the day of preparation although it was found that storage for 36 hours at 4.0° C. did not affect the results.

Filter Paper Strips.—Sterile strips of filter paper (Whatman No. 42), 87 mm. by 6 mm., were cut and soaked in the appropriate antibiotic solution. The excess fluid was allowed to drain off and the strips were then placed across the central portion of the gradient plates, following inoculation of the plates with the test organism.

Inoculum.—The bacterial inoculum consisted of 6 drops of a 6-hour digest broth culture added to 3 ml. melted and cooled 1.0 per cent nutrient agar. The agar was then poured across the surface of the plate and allowed to solidify. Most of the tests were carried out with *Staphylococcus aureus* (Oxford strain). A number of other organisms were also tested and gave identical results with the antibiotics to which they were sensitive (*Salmonella typhi, Escherichia coli,* etc.).

All plates were incubated overnight at 37.0° C. and the results noted. With each organism a control gradient plate was included, omitting the antibiotic, to make certain that growth was equal and satisfactory over the pH range.

RESULTS

Figs. 1 to 10 show the patterns of inhibition produced with the various antibiotics and with varying concentrations of the same antibiotics.

1. *Comparison of Different Antibiotics.*—It will be seen that the various antibiotics fall into three main groups according to the nature of the zone of inhibition.

Group 1 (Figs. 1 and 2) consists of penicillin and chloramphenicol. Here the degree of diffusion is more or less equal over the whole pH range so that the growing edge of the inoculum is parallel to the antibiotic strip. At pH levels below that of the gradient plates penicillin was found to have a more marked effect since it is a fairly strong acid. In the case of chloramphenicol (Fig. 2) there is an appearance of a larger zone of inhibition at both edges of the plate. This appearance was noted with other antibiotics and is probably the result of two factors; (1) the buffering effect is poor at the edge of the plate and (2) the thickness of the agar is less than in the rest of the plate due to the curvature of the bottom. This latter point was investigated further by preparing agar plates with the agar sloped across the plate in a wedge shape. Antibiotics strips were then placed on the surface as described above and the plates inoculated. Diffusion was found to be considerably more marked in the thin section of the agar.

Group 2 (Figs. 3 to 9) consists of those antibiotics whose effect is most marked on the alkaline side, i.e., streptomycin, erythromycin, and spiramycin. Group 3 is represented by chlortetracycline (Fig. 10) where the maximal effect is on the acid side. Tetracycline and oxytetracycline were found to give similar patterns of inhibition to chlortetracycline.

2. *Effect of Varying Concentrations of Antibiotic.*—A comparison of Figs. 3 and 4; 5, 6, and 7; and 8 and 9 shows the effect of varying the concentration of antibiotic. With those drugs whose effect is most marked on the alkaline side, the effect of decreasing concentrations is to increase the pH level at which

the end point of no inhibition of growth is apparent. With increasing concentration there appears to be a direct relationship between the percentage inactivation of antibiotic and its concentration, at any particular pH level.

Fig. 1. Fig. 2.

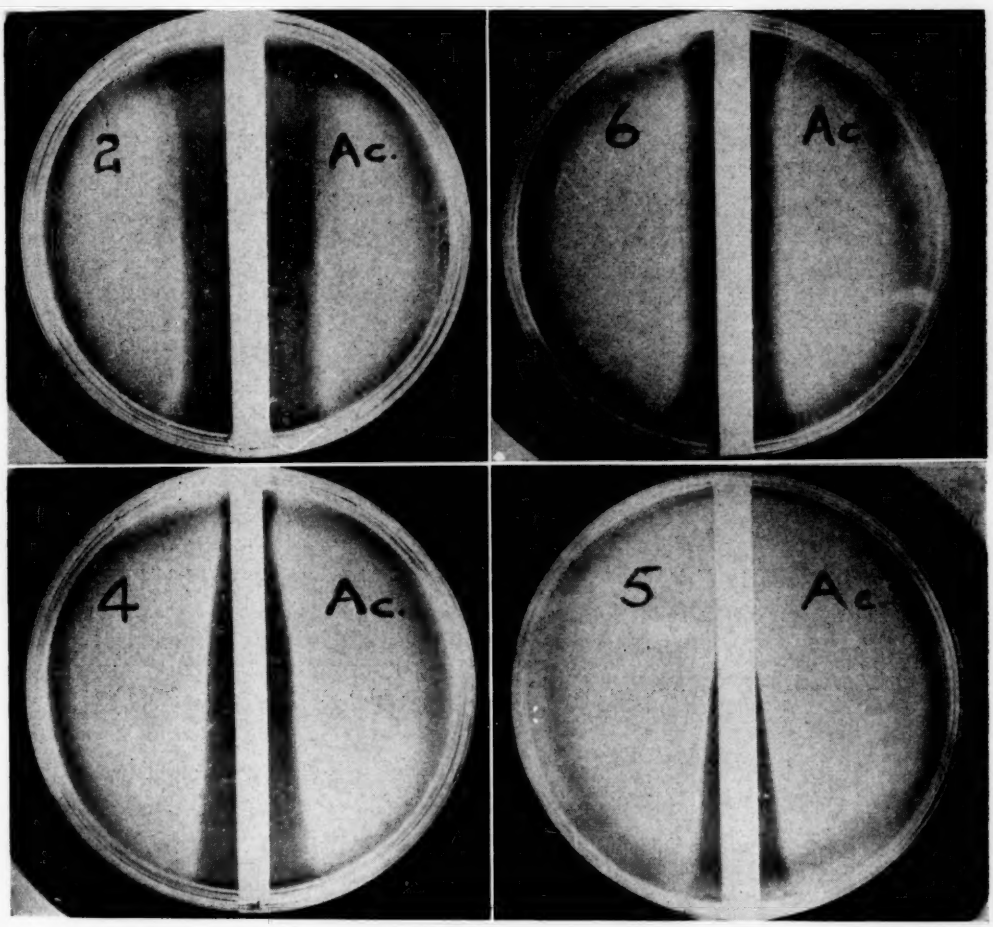

Fig. 3. Fig. 4.

Fig. 1.—Effect of penicillin (100 units per milliliter) incorporated in strip. Ac = acid side of plate. There is practically no difference in the zone of inhibition in the acid and alkaline regions. Test organism, *Staph. aureus* var. *Oxford.*

Fig. 2.—Chloramphenicol at a concentration of 1,000 μg/ml. Here the zone of inhibition is considerably narrower than in Fig. 1 but otherwise has similar features. Test organism, *Staph. aureus* var. *Oxford.*

Figs. 3 and 4.—The antibiotic strip in Fig. 3 contains 5,000 μg/ml. of streptomycin sulphate and 1,000 μg/ml. in Fig. 4. Fig. 4 shows complete resistance of the test organism (*Staph. aureus* var. *Oxford*) in the acid region.

However, changes in the concentration do not appear to affect the size of the angle of the zone of inhibition from the strip. This is particularly well shown in Figs. 5 and 6 where a tenfold increase in concentration has merely altered the pH level at which no inhibition of growth becomes apparent.

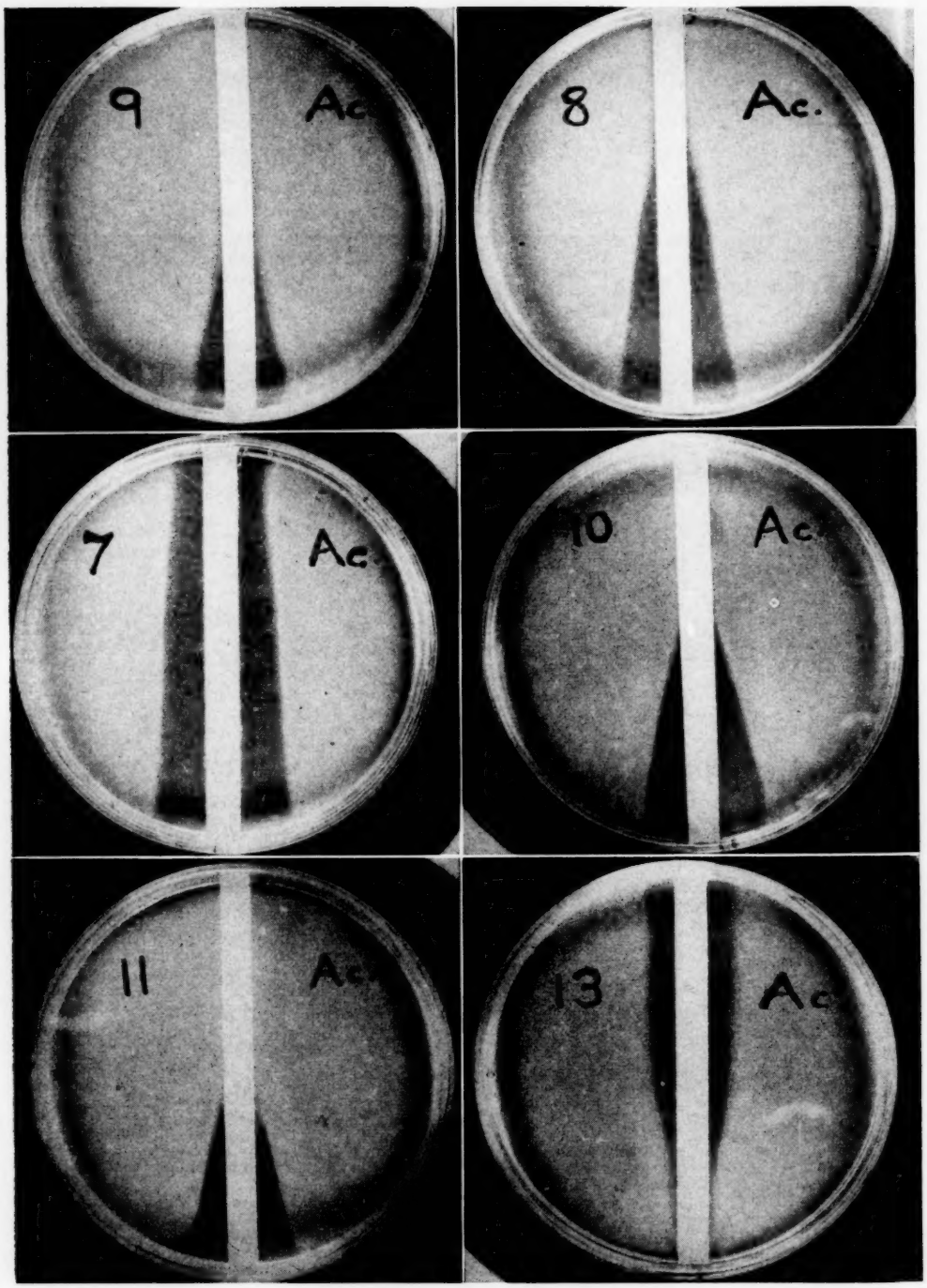

Fig. 5.

Fig. 7.

Fig. 9.

Figs. 5, 6, and 7.—Fig. 5 shows the effect of erythromycin at a concentration of 10 μg/ml. against the test organism (*Staph. aureus* var. *Oxford*). Concentration of erythromycin in Fig. 6 is 100 μg/ml. and in Fig. 7 1,000 μg/ml. Again the marked increase in activity in the alkaline zone is well seen.

Figs. 8 and 9.—The effect of spiramycin at 500 μg/ml. (Fig. 8) and at 100 μg/ml. (Fig. 9). The pattern of inhibition is similar to that shown by erythromycin. Test organism, *Staph. aureus* var. *Oxford*.

Fig. 10.—Chlortetracycline at a concentration of 100 μg/ml. Here there is inhibition of antibiotic activity in the alkaline region. Oxytetracycline and tetracycline gave identical results. Test organism, *Staph. aureus* var. *Oxford*.

With penicillin and chloramphenicol the effect of altering the concentration was merely to narrow or widen the zone of inhibition which remained parallel to the strip.

The angle of the zone of inhibition from the strip is not constant for the various antibiotics. A comparison of Figs. 4 and 8 shows the type of inhibition with streptomycin and spiramycin. In both, the zone of inhibition commences at approximately pH 6.6 but the angle of inhibition on the alkaline side is considerably more obtuse with spiramycin than it is with streptomycin. Similarly, the type of inhibition with erythromycin is more closely related to that of spiramycin than to streptomycin. Two explanations appeared possible for this effect. Either the percentage inactivation of the antibiotic with each rise in pH, was greater in the case of streptomycin than for a corresponding rise with the other two drugs, or, if the percentage inactivation was identical at any particular pH, the differences in the patterns of inhibition might merely be related to differences in the diffusibility of the antibiotic.

3. *Effect of Hydrogen-ion Concentration on Percentage Inactivation of Antibiotic.*—To determine which of the above possibilities seemed to be the correct explanation, minimum inhibitory concentrations of streptomycin, spiramycin, and erythromycin were determined at pH 6.0 and at pH 8.0 by a tube dilution method. A comparison of the end points at the different pH levels showed that the ratio of the minimum inhibitory concentrations was identical for both antibiotics. These results suggest that the percentage inactivation with decrease in pH is similar for both drugs. The differences in the angles of inhibition would seem to be dependent on differences in the diffusibility of the antibiotics. It is not possible to correlate this with molecular size since the exact structure of the spiramycin molecule is at present unknown. Erythromycin, however, has a molecular weight of 733 as compared with streptomycin sulphate which is 1,457.

4. *Effect of Hydrogen-ion Concentration on Antibiotic Strips.*—Further investigations were carried out to determine what effect the hydrogen-ion concentration of the agar had on the antibiotic incorporated in the strips. Plates were prepared as described and suitable concentrations of antibiotic employed such that the zones of inhibition were limited to the alkaline side (in the case of streptomycin, erythromycin, and spiramycin) and to the acid side in the case of the tetracyclines. After overnight incubation the strips were removed from the agar and placed across the surface of fresh agar plates of constant pH 7.4. These plates were inoculated with the test organism and incubated at 37.0° C. Control strips which had been incubated overnight at 37.0° C. were similarly placed on inoculated plates of pH 7.4. These control strips still produced satisfactory zones of inhibition showing that their potency had been unaffected by the initial overnight incubation.

In the case of the strips transferred from the streptomycin, erythromycin, and spiramycin plates, however, there were no zones of inhibition in relation to those parts of the strips which had been in contact with the acid side of the gradient pH plates. Similarly, there was growth right up to the edge of the

strips where they had been in contact with the alkaline side of the agar, as in the case of the tetracycline drugs. The effect of an unsatisfactory hydrogen-ion concentration was thus to inactivate the antibiotic and not merely to hinder its diffusion.

DISCUSSION

The very wide differences which exist in the optimal hydrogen-ion concentration for activity in the case of the different antibiotics, indicates clearly the importance of strict control of the pH of the medium employed.

False impressions may otherwise be obtained regarding the likely effect of an antibiotic in vivo. This is particularly the case where the concentrations employed in sensitivity tests in vitro bear little relation to the concentrations which are attainable in the blood and tissue fluids.

Among the basic antibiotics other than streptomycin, both streptothricin and proactinomycin are also more active with increase of pH. In the case of the latter for example Geiger and associates[3] have shown that an increase of pH from 6.5 to 7.5 has an effect equivalent to a fourfold increase in the concentration of the antibiotic. With regard to penicillin, Stokes[4] states that the zones of inhibition do not vary between pH 6.0 and 8.0. This agrees with our observations (Fig. 1). Foster and Woodruff,[5] however, found that a shift of pH from 7.0 to 5.5 resulted in an apparent threefold increase in effect in the case of penicillin.

The choice of an antibiotic in the control of a focus of infection depends on factors involving host-parasite and host-drug relationships in addition to the demonstration of sensitivity of the causal organism. Changes in hydrogen-ion concentration in various parts of the body, for example, may be great enough to influence the activity of the antibiotic. In the case of a urinary infection it is obviously essential to render the urine alkaline if streptomycin is to be given. Such a procedure would be contraindicated, however, if the tetracycline drugs were to be employed. Similarly, where there is a focus of necrotic tissue, produced as the result of infection, the pH of the region tends to be highly acid. In such a situation a drug of the tetracycline group may prove to be more effective than one whose optimal activity lies on the alkaline side. In the case of systemic infections where the effect of the antibiotic is dependent on its activity in the blood and tissue fluids at pH of approximately 7.4, it would seem that relatively greater amounts of the tetracycline drugs would be required than in the case of streptomycin, erythromycin or spiramycin. The relative ineffectiveness of the tetracycline drugs in typhoid fever as compared with chloramphenicol may partly be explained by the fact that the pH of the tissue fluids and blood will tend to decrease the activity of the former. In vitro estimations show that the minimum inhibitory concentrations of these antibiotics for *S. typhi* are very similar, indicating that host factors are of great importance in determining the effect of antibiotics in vivo.

The principle of the gradient-type plate has been applied to other types of investigation. Sacks[2] made use of it to study the effect of varying pH levels

on the growth of organisms. Streitfeld and Saslaw[6] have described a modification for determining the sensitivity of organisms to paired combinations of antibiotics.

SUMMARY

1. The effect of antibiotics, at varying hydrogen-ion concentrations on *Staphylococcus aureus* (var. *Oxford*) is described. The method employed consisted of a gradient-type pH plate.

2. The results indicate the importance of adequate control of pH in media which is to be used for determining sensitivity of organisms to antibiotics.

We are indebted to May & Baker (S.A.) for a supply of pure spiramycin and to Parke, Davis & Co., for the pure chloramphenicol.

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LABORATORY METHODS

NEUTRON ACTIVATION ANALYSIS OF SODIUM IN BLOOD SERUM

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NEUTRON activation analysis offers an essentially nondestructive means of quantitatively estimating certain anions and cations. As part of an investigative program on the applications of neutron activation analysis to biology and medicine,[1] this laboratory has studied the determination of sodium in blood. The procedure has been found to be rapid and reproducible, and has the added advantage of entailing minimal handling of the specimen. Since the material is not consumed, it may be reassayed or used for other studies.

The steps involved in the operation consist of: irradiation of a sample in a low intensity neutron flux, measurement of the induced radioactivity, and comparison of the radioactivity with that of a standard handled in identical fashion. This report describes the method and the results obtained after "contaminating" short half-life isotopes have decayed.

PRINCIPLE

When tissue is exposed to a neutron flux, interactions occur between bombarding particles and target nuclei. With fast neutrons, multiple nuclear events, such as (n,γ), (n,p), and (n, 2n), can occur. However, when thermal neutrons are employed, they almost exclusively engender (n,γ) interactions, and other processes may be neglected. Following thermal neutron bombardment, radioactivity induced in any particular element within the specimen is given by the expression[1]:

$$M = \frac{(1.63 \times 10^{-5})\ n\ .\ \sigma A\ .\ W\ .\ f\ .\ (1 - e^{-\lambda t_1})\ (e^{-\lambda t_2})}{A_t}\ . \qquad 1.$$

M is the activity in microcuries; n the flux in neutrons per square centimeter per second; σ A the isotopic neutron capture cross section in barns; W the weight of the element in grams; f the fractional abundance of the precursor isotope in the element; A_t the atomic weight of the element; λ the disintegration constant of the product radioactive isotope; t_1 the time of sample exposure to the neutron flux; and t_2 the interval elapsed from the cessation of irradiation to the beginning of analysis.

Equation 1 can be solved for the neutron flux ($n = M.A_t/$ etc.). Hence if a material of known composition and an unknown are exposed to the same flux, they may be

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equated. If both samples were subjected to an identical flux for a similar time interval, and then analyzed simultaneously, they would be related by the expression:

$$\frac{Ms}{Ws} = \frac{Mu}{Wu} \qquad 2.$$

where M is the radioactivity, W is the weight of the element, s represents the standard, and u represents the unknown. In other words, the radioactivity induced under such conditions would be proportional to the quantity of material originally present.

When only one device for detecting radioactivity is present, the standard is analyzed first, and the unknown is usually counted subsequently. In this situation, the more general one, activity in the standard must be corrected to the time the unknown is counted.

$$\frac{Ms\ (e^{-\lambda\ t_0})}{Ws} = \frac{Mu}{Wu}. \qquad 3.$$

The time elapsed between the onset of analysis of the standard, and the beginning of the same procedure on the unknown, is represented by t_0.

Equation 3 is the mathematical expression used in the present analysis. The measurement of the radioactivity present may be performed by any suitable device, and expressed in any units, as long as the same method is utilized for the standard and the unknown. Two techniques for determining radioactivity were employed: counting in a well-type scintillation counter, and utilizing the area under the gamma ray spectral curve.

METHODOLOGY

Samples consisted of dried human serum of known composition.* Polyethylene tubes, whose outer dimensions measured 15 × 35 mm., were washed in nitric acid and distilled water, then air dried. Each sample was placed in one of these containers, and heat sealed. Tubes were again washed, to remove contaminants resulting from handling.

Loaded tubes were placed in a polyethylene bag and lowered into the glory hole of a pool-type nuclear reactor.† Flux densities varied from 2×10^7 neutrons/cm.^{2}/sec., to 2×10^9. Reasons for this choice of values (which include their availability from small nuclear reactors, Van de Graaff—beryllium sources and radium—beryllium systems) have been presented elsewhere.[1] Time of irradiation was 20 to 60 minutes, depending upon the flux.

The actual value of the neutron flux can be calculated by inserting a known weight of sodium or other foil into the reactor, measuring the induced radioactivity after a known period of time, and substituting in equation 1. Practically however, this step is of no consequence, since standard and sample are both subjected to the same flux.

After irradiation, samples were counted in a well-type scintillation counter, and then further analyzed in a Nuclear Model 1810 pulse height analyzer and well-type crystal. The base line voltage of the analyzer was continuously varied by a motorized potentiometer. Pulses within 1.0 volts above the base line were fed through a scaler and ratemeter to a rectilinear recording galvanometer. The potentiometer was driven at a rate of 1 turn/50 minutes. The time axis was also the axis of base-line voltage, and a distance of 10 inches on the recording paper corresponded to scanning from 0 MEV to 4 MEV.

The area under the gamma ray curve, rather than peak height measurement, was utilized for estimating the quantity of radioactivity. This was done for two reasons. First the height of a particular peak might partially be due to the photo-emission of a contaminant. When averaged over a larger total area such a contribution becomes negligible. Second, when using a narrow discrimination (a window only one volt wide) the peak height may not be perfectly linear; this must be checked for each system used.

"Noise" on the gamma ray spectrometer is low. As long as the 1 K or a higher scale is used on the count rate meter, no correction is necessary for background recorded

*Versatol, Warner-Chilcott, Laboratory Supply Division, Morris Plains, N. J.

†We are indebted to the Staff of the Nuclear Reactor Project, Naval Research Laboratory, Anacostia, Md., for the service irradiations.

by the gamma ray spectral curve. When the 300 scale is employed, a background correction is necessary. Radioactivity induced in the polyethylene tubes was negligible, and possessed a short half-life (perhaps Cl^{38}). No counts from the container registered at the sodium photoelectric peak.

To calibrate the apparatus, Na^{24} of known activity (purchased from the A.E.C.) was utilized. The system was also calibrated for internal stability and reproducibility, by introducing a small concentration (about 0.1 microcurie) of Na^{24}, and obtaining repeated gamma ray spectral curves. The area under each 50-minute tracing should have been 0.963 times the area under the preceding curve (0.963 represents the decay of Na^{24} during 50 minutes). In Table I are listed the measured, as well as the theoretically calculated areas during a trial run. The error in calculating the successive areas by use of a planimeter (vide infra) was below 2 per cent.

TABLE I. AREA BENEATH GAMMA RAY SPECTRUM OF A SAMPLE OF Na^{24}, OBTAINED BY HAVING SPECTROMETER TRACE REPEATED 50-MINUTE CURVES

TIME IN MINUTES	AREA IN SQUARE INCHES THEORETICAL	MEASURED	DIFFERENCE IN SQUARE INCHES	DIFFERENCE IN PER CENT
0- 50	—	7.81	—	—
50-100	$7.81 \times (0.963)^1 = 7.52$	7.41	0.11	1.5
100-150	$7.81 \times (0.963)^2 = 7.24$	7.29	0.05	0.7
150-200	$7.81 \times (0.963)^3 = 6.97$	6.90	0.07	1.0
200-250	$7.81 \times (0.963)^4 = 6.71$	6.63	0.08	1.2
250-300	$7.81 \times (0.963)^5 = 6.46$	6.40	0.06	1.0

Theoretical calculations[1] have indicated that exposure of 33 mg. sodium (the approximate quantity in 10 ml. serum) to a flux of 2.5×10^8 neutrons/cm.^{2}/sec. for a period of one hour, will result in the production of 0.14 μc of Na^{24}. To test the ability of the recording system to distinguish between different quantities of radiosodium, quantities of the isotope varying from 0.05 to 0.35 μc were employed.

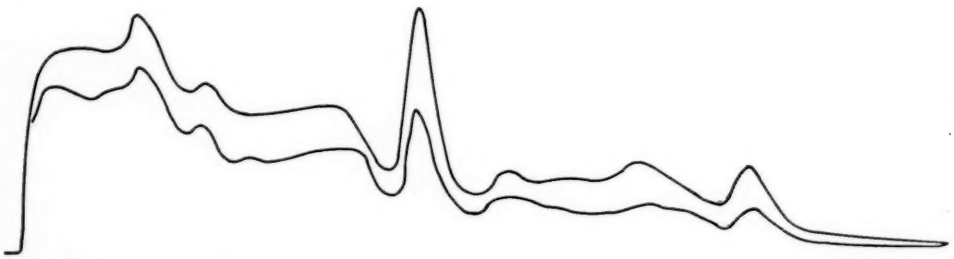

Fig. 1.—Superimposed gamma ray spectra from two samples of radiosodium (Na^{24}). First upsweep corresponds to zero MEV. Last major peak is from the 2.75 MEV gamma ray. See text for details.

A result of such a study is shown in Fig. 1. The lower curve represents the spectrum obtained utilizing 0.070 μc Na^{24}. Instrument settings were: analyzer: gain 1, 1 volt window; count rate meter: 10 second response, 10 K scale. By means of a Keuffel and Esser (4236) compensating polar planimeter, the area under the curve was determined. The "cutoff point" on the graphs has been considered to be the end of the photoelectric peak corresponding to the 2.75 MEV gamma ray. It must be recognized that decay was also occurring throughout the 50-minute scan, but this is neglected in subsequent calculations since the same decay was occurring during the tracing of later samples. That is:

$$\text{Amount in A (Decay during 50 minutes)} = \frac{\text{Y (Amount in B)}}{\text{(Decay during 50 minutes)}}. \qquad 4.$$

For the particular curve illustrated, the area was 3.89 square inches. Fifty minutes later, due to radioactive decay, the area traced would have theoretically been:

$$A = A_0\, e^{-\lambda t}$$
$$A = (3.89)\ (e^{-0.0462 \times 0.833})$$
$$A = 3.74 \text{ square inches.}$$

The factor 0.0462 is the disintegration constant of Na^{24} expressed in reciprocal hours, and 0.833 is the decimal equivalent of 50/60 hours.

The upper curve in Fig. 1 (enclosing 7.35 square inches) is the spectrum from 0.14 μc Na^{24}, run 50 minutes after the first tracing. Twice the corrected first area (representing 0.07 μc) should equal the area under the second curve (representing 0.14 μc). This it does, within $\frac{7.35}{2 \times 3.74} \times 100$, or 98.2 per cent. Errors in pipetting, recording radioactivity by the spectrometer, and determining the area with a planimeter, were less than 2 per cent. On repeated trials, the error has not exceeded 3 per cent.

When pure samples are employed, counting in a well-type scintillation counter is more convenient and expedient than use of a gamma ray spectrometer. However, if there is uncertainty as to the number of radioactive constituents in a sample, gamma ray spectral curves are essential. Hence, in the initial experimental work, a spectrometer was employed. This method is applicable to any gamma emitting isotope. As will be shown, 12 hours after irradiation of blood serum, Na^{24} is the only major gamma ray emitting isotope remaining.

RESULTS

After one hour bombardment of 10 ml. serum of normal composition by a flux of 2.5×10^8 neutrons/cm.^{2}/sec., the following gamma ray emitting isotopes are present[1] to the extent of 30 disintegrations per minute (d.p.m.) or greater: Na^{24}, Cl^{38}, Br^{80}, Br^{80m}, Br^{82}, I^{128}, K^{42}, Ca^{49}, Cu^{64}, Cu^{66}, Mg^{27}, and depending upon the initial concentration, perhaps Co^{60m} and Mn^{56} (m refers to the first of two metastable isomers). Thus, the area under the gamma ray curve immediately after neutron bombardment, contains contributions from a multitude of radionuclides. The general shape of the curve is that of pure Na^{24}, although its area is not determined by radiosodium alone. Estimation of the quantity of sodium originally present in the specimen cannot be accurately performed by estimating the area bounded by the curve, since that quantity is a complex representation of many isotopes.

TABLE II. GAMMA EMITTING ISOTOPES REMAINING 12 HOURS AFTER BOMBARDMENT OF 10 ML. SERUM* BY 2.5×10^8 NEUTRONS/CM.^{2}/SEC., FOR ONE HOUR*

ISOTOPE	HALF-LIFE IN HOURS	GAMMA† FACTOR	D.P.M. AT T 12‡	TOTAL GAMMA/MIN.	RATIO: $100 \times$ GAMMA/MIN. ISOTOPE / GAMMA/MIN. Na^{24}
Na^{24}	15.0	2.00	184,640	369,280	100.00
Cl^{38}	0.62	0.47	2	1	0.00
Br^{80m}	4.6	2.00	388	776	0.21
Br^{82}	35.9	1.00§	215	215	0.06
K^{42}	12.5	0.20	983	196	0.05
Cu^{64}	12.8	0.38‖	116	44	0.01

$$\frac{Na^{24} \text{ gamma/min.}}{\text{Total gamma/min.}} = \frac{369{,}280}{370{,}512} \text{ or } 99.6 \text{ per cent}$$

*Assumed composition of the serum: sodium, 33 mg.; chlorine, 34 mg.; bromine, 0.1 mg.; potassium, 1.6 mg.; copper, 10^{-2} mg.

†Average number of gamma rays per disintegration.

‡Disintegrations per minute, 12 hours after removal of the sample from neutron flux.

§Assumed, value may be in error.

‖Due entirely to positron annihilation. Br^{80m} refers to the first of two metastable forms.

Twelve hours after removal of the sample from the neutron flux, the "contaminating" nuclides have markedly decreased (Table II). From the Table, it can be observed that gamma rays from Na^{24} constitute nearly 99.6 per cent of the gamma emissions from the irradiated blood serum. The quantity of sodium originally present in the sample may then reasonably be calculated by determining the radioactivity at this time, and substituting into equation 3.

Any suitable detection device could have been used to count the 12-hour specimen. Use of a well-type scintillation counter gave good results. A gamma ray spectral curve was obtained on this specimen simply to rule out the possibility of a contaminant being present. As an example: twenty-one hours after the exposure of 10 ml. dried human serum (containing 32.5 mg. sodium) to a flux of 1.29×10^9 neutrons/cm.^{2}/sec. for thirty minutes, the gamma ray spectrum was obtained. Instrument characteristics were: analyzer: 1 volt window, gain 1; count rate meter: 2 second response, 3 K scale. Area under the curve was 5.88 square inches. An "unknown" that had been subjected to the same flux for an identical period, was then (50 minutes after the start of the first sample) placed in the gamma ray spectrometer. Area under this curve was 5.18 square inches. By use of Equation 3:

$$\frac{5.88\ (e^{-0.0462 \times 0.833})}{32.5\ \text{mg.}} = \frac{5.18}{\text{Wu}}$$

Wu = 29.7 mg. Actual weight of sodium in the unknown sample was 30.1 mg. (determined by the manufacturer, employing flame photometry). Agreement between the values obtained by activation analysis and by flame photometry was $\frac{29.7}{30.1} \times 100$ or 98.7 per cent.

Presented in Table III are six consecutive analyses of sodium in dried blood serum as obtained by activation, and the corresponding value by flame photometry. Difference between the two techniques was less than 3 per cent.

TABLE III. ESTIMATION OF SODIUM IN BLOOD SERUM BY NEUTRON ACTIVATION ANALYSIS, AS COMPARED WITH FLAME PHOTOMETRY. SAMPLES RUN 12 TO 21 HOURS AFTER COMPLETION OF IRRADIATION

SODIUM IN MG.		DIFFERENCE	
FLAME PHOTOMETRY	ACTIVATION ANALYSIS	IN MG.	IN PER CENT
16.3	16.5	0.2	1.2
20.8	20.3	0.5	2.4
25.9	26.3	0.4	1.5
30.1	29.7	0.4	1.3
30.4	30.1	0.3	1.0
32.5	32.9	0.4	1.2

DISCUSSION

Neutron activation analysis is an essentially nondestructive analytic procedure. Even if the neutron flux induced protein denaturation, there would be no interference with the analysis of radioanions and radiocations, as long as the entire specimen was counted. An infinitesimal number of nuclei, when compared with the total number in the sample, are made radioactive by neutron

bombardment. Hence, the material may be assayed repeatedly, or used for other tests. A sodium analysis can be performed on blood serum, and the sample will still be intact for later studies. Studies have also been performed using liquid plasma and serum. Aside from the fact that they require more space in the nuclear reactor, they give results identical to those obtained from dried serum.

The predominance of the Na^{24} gamma emission may be taken advantage of in additional ways. Thus, by shielding the sample, to reduce the activity of other gamma rays, and counting at the 2.75 MEV photoelectric peak, it is possible to obtain an estimation of the quantity of radiosodium after a period of irradiation as short as ten minutes. Our experience with this is limited but has been promising. Reiffel and Stone[2] used a somewhat similar technique for estimating sodium in muscle. If sodium is but one of the electrolytes to be quantitated in serum, the irradiated sample may be fractionated by means of suitable anion and cation exchange resins. This technique has proved useful, and is being investigated further.

The gamma ray spectrometer—area technique, as discussed, is applicable to any gamma emitting isotope. The procedure is of great value when a radiocontaminant is suspected. First, the gamma ray peak characteristic of the foreign material may perhaps be detected. Second, the area beneath the curve should diminish, on successive tracings, according to the decay scheme of the isotope studied. If it does not, a radiocontaminant is present.

Two techniques may shorten the counting time required. These are: use of a multichannel analyzer (or counting at the desired photoelectric peak), and the employment of a scintillation well-type counter. The multichannel analyzer rapidly produces the desired scan. If counting is performed only at one photoelectric peak, care must be taken that the response is truly linear at this point; if not, a calibration curve must be constructed. Well-type scintillation counting is readily performed, but it entails the danger of detecting counts from contaminants. Still another approach to the problem is to separate sodium from the other blood constituents, either before or after irradiation.

In addition to sodium, isotopes of chlorine, bromine, iodine, potassium, calcium, copper, magnesium, and possibly cobalt and manganese are produced to the extent of 30 d.p.m. or greater by the exposure of 10 ml. serum to a flux of 2.5×10^8 neutrons/cm.^{2}/sec. for one hour. Quantitative estimation of these materials is theoretically possible if complete separation were possible. Erythrocytes, with a low sodium content, may be directly analyzed by means of neutron activation analysis, and data will be presented in a later report.

SUMMARY

1. A relationship is derived indicating that radioactivity induced in an element common to a standard and an unknown, following identical neutron bombardment, is proportional to the weight of material originally present in each, if correction is made for the time difference between the analyses.

2. Twelve hours after irradiation of the solid material from 10 ml. serum, by a flux of 2.5×10^8 neutrons/cm.^{2}/sec. for one hour, 99.6 per cent of the gamma emissions present are due to Na^{24}.

3. Utilizing the amount of radioactivity in the 12-hour postirradiation specimen, the quantity of sodium present in serum may be estimated. Results agree within three per cent with values obtained by flame photometry.

4. A technique of estimating the area beneath the gamma ray spectral curve of an isotope is described, and its use discussed in detecting radiocontaminants or estimating the quantity of activity.

5. It is concluded that neutron activation analysis may be used to determine sodium in blood serum. The method is essentially nondestructive, and may be performed on samples which must be saved for other determinations. Extensions of the procedure are mentioned, including a technique for the rapid estimation of sodium, and one for the fractionation of serum into its component radioanions and radiocations.

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A WELL SCINTILLATION COUNTER FOR IMPROVED VOLUME EFFICIENCY

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IN THE use of a well scintillation counter to assay radioactive samples for various clinical tests, it has become increasingly important to employ volumes in the range of 2 to 10 ml. Various techniques involving the determination of plasma levels of protein-bound I^{131} after the administration of tracer doses have indicated the advantage of utilizing these larger samples. Two typical techniques used in this laboratory for such determinations include the method of Morton[1] for separating butanol extractable I^{131} from plasma and the method of Lahr and Tabern[2] for separating protein-bound I^{131} from the plasma iodide by ion exchange resin. For low-level determinations it has been desirable to determine the radioactivity derived from at least a 4 ml. plasma sample, which has entailed analyzing a volume of 8 ml. for the butanol extraction method and 7 ml. when the resin technique is employed.

In addition there are various samples which contain the radioactivity in a nonhomogenous distribution that may vary during the period of assay. An example of this is the measurement of whole blood volume by such agents as iodinated (I^{131}) serum albumin or Cr^{51}-tagged red cells. When these whole blood samples are assayed in a well scintillation counter the red cells will, of course, settle to the bottom of the tube while the serum is rising to the top. This presents a continually changing sample geometry to the well counter that can be avoided only by taking short period assays or having a well counter relatively independent of sample volume or distribution.

Since the conventional commercially available thallium activated sodium iodide well crystal exhibits a loss of efficiency for samples much over 2 ml., it would seem desirable to construct a well counter capable of handling larger volumes and exhibiting much less dependency on sample volume or distribution. This has been approximated by the well counter of Baskin, Demorest, and Sandhaus[3] which showed both decreased volume dependency and the ability to handle volumes of 20 ml. or more. This counter, however, is not as efficient as the conventional well crystal until the sample volume exceeds 5 ml. Since many applications will still employ volumes less than 5 ml. there is a reluctance to lose the greater efficiency of the smaller well counter. For this reason a modification in these well dimensions has been made so that the larger well will exhibit the same absolute efficiency as the smaller well, handle larger volumes, and show decreased volume dependency.

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EQUIPMENT

For the larger well we have utilized a sealed thallium activated sodium iodide crystal measuring 2 in. in diameter and 2 in. in height with a well 1⅛ in. in diameter and 1½ in. deep. The outer diameter of this crystal is small enough to be immediately interchangeable with almost all probe units employing RCA 5819 or Dumont 6292 photomultiplier tubes. This well diameter has provided ample room for a brass protective liner in the well while still seating a standard 24 mm. diameter glass vial. The vials utilized were 82 mm. in height and hold approximately 24 ml. These vials* are relatively inexpensive and are specified by the manufacturer to be accurate in diameter to within 0.5 mm. Fig. 1 shows the crystal, brass liner, and vials.

Fig. 1.—Large well crystal with brass protective cap and sample vials.

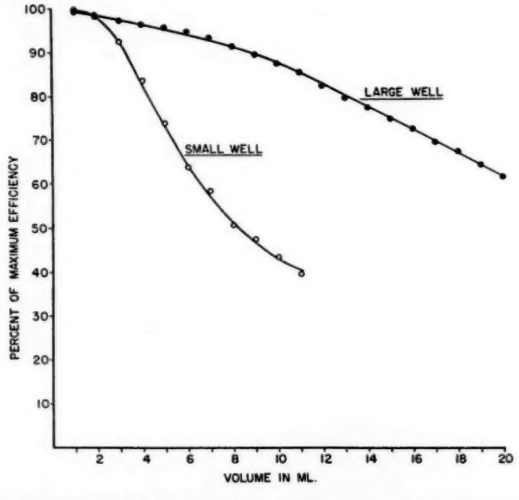

Fig. 2.—Comparison of relative counting efficiences of variously sized well crystals with respect to sample volume. All samples normalized to the response of the small well crystal to a 0/0.01 μc sample of I^{131}.

The conventional thallium activated sodium iodide crystal† measured 1¾ in. in diameter and 2 in. in height with a hole ¾ in. in diameter and 1½ in. deep. Klett colorimeter test tubes provide a close fit for this well and have been selected for sample analysis.

*Titeseal vials, The Chemical Rubber Co., Cleveland, Ohio.
†Sealed unit provided by the Harshaw Chemical Company, Cleveland, Ohio.

Both well counters were shielded on all sides with approximately 2 in. of lead for a total height of 9 in. Background of the smaller well counter averaged 250 c.p.m. as compared to 350 c.p.m. for the larger unit. The preamplifiers, photomultiplier tubes, and scalers used with each exhibited similar characteristics throughout.

RESULTS

A comparison of the relative counting efficiencies of these variously sized well counters with respect to sample volume is shown in Fig. 2. The response of the conventional well counter to a 0.01 μc sample of I^{131} in a volume of 0.5 ml. has been normalized to 100 per cent. The fall off of efficiency as the same activity is assayed in successively larger volumes is then a reflection of both lesser geometric efficiency as well as the self-absorption of the gamma rays in the liquid itself.

For practical clinical assays the advantages of the larger unit are shown in Table I. Relative counting rates as obtained from a 6 ml. and an 8 ml. whole blood samples from a patient injected with 10 μc of iodinated (I^{131}) serum albumin are shown as counted with both the smaller and larger well crystals.

TABLE I. COMPARISON OF RELATIVE EFFICIENCY AND VOLUME DEPENDENCY FOR WHOLE BLOOD ASSAY FOLLOWING 10 μC INTRAVENOUS IODINATED (I^{131}) SERUM ALBUMIN ADMINISTRATION

6 ML. WHOLE BLOOD SAMPLE	SMALL WELL (C.P.M.)	LARGE WELL (C.P.M.)
Counted immediately	12,600	18,750
After standing ½ hour	11,350	18,450
After centrifuging	8,900	18,200
8 Ml. Whole Blood Sample		
Counted immediately	13,850	24,200
After standing ½ hour	12,200	23,700
After centrifuging	7,800	23,000

DISCUSSION

The advantage of improved efficiency is shown in Table I in which it is noted that while only a small increase in counting rate can be obtained by increasing sample volume from 6 ml. to 8 ml. in the small well counter, the larger well exhibits an increase of about one-third in counting rate for a similar increase in volume. The second advantage observed is the volume independence of the latter unit indicated in the counting rates of the same blood sample after standing 30 minutes and after centrifuging. At this time the red cells have predominantly settled to the bottom and the plasma risen to the top, dependent upon sedimentation rate and hematocrit. In the patient cited the hematocrit was 39 per cent and the sedimentation rate was 12 mm./hr. Since the volume dependence of the small unit is fairly critical this separation has caused a deviation as large as 10 per cent after standing while the larger unit has shown essentially negligible variation.

SUMMARY

1. An improved well scintillation counter has been designed for counting radioisotope samples in volumes of 3 to 20 ml. with an appreciable increase in efficiency over the conventional well counters used with these volumes.

2. The relative volume independence of this unit has indicated its usefulness in producing a stable assay with samples which contain the radioactivity in a nonhomogenous distribution that may vary during the period of measurement.

We wish to thank Miss June Dockery for her assistance in these measurements.

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STUDY OF PAPER ELECTROPHORESIS: COMPARISON WITH SALT PRECIPITATION OF SERUM PROTEIN FRACTIONS

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WHEN the Spinco Model R paper electrophoresis apparatus was introduced into our laboratory, we compared this technique with our standard procedure of protein fractionation, a modification of the relatively simple Wolfson and Cohn[1] method. In addition, the recoveries of known solutions of albumin and gamma globulin and the "tailing" effect of albumin in the paper electrophoresis system were determined. The present paper describes these studies, emphasizing the comparison of the clinical methods of protein fractionation.

MATERIALS AND METHODS

Approximately 50 serum specimens were obtained from professional blood donors. At the end of the blood donation, approximately 10 ml. of blood was collected into a chemically clean test tube; the blood was expressed from the tubing through the needle used in the collecting set. The specimens were centrifuged and the sera placed in the refrigerator overnight.

A control serum was obtained by collecting a unit of clotted blood from one professional donor. The serum was separated under sterile conditions and aliquots transferred to sterile, plain Vacutainer tubes† which were stored in a Deepfreeze. On the day of collection, control values for both methods of protein fractionation were established by quadruplicate determinations. There was no noticeable change in the protein fractions of this control serum when aliquots were later removed and thawed just before using.

Both methods of protein fractionation were done in duplicate on each serum simultaneously to avoid possible differences between the two methods; e.g., length of time in the refrigerator, temperature changes, and time elapsed between collection and determination. We employed the acceptable 6-hour accelerated electrophoretic separation[2] (which compared very well with the usual 16-hour separation) so that both chemical precipitation and paper electrophoresis could be instituted at the same time. We added 6 lambda of the serum or protein solution to the paper strips; this volume contained sufficient protein for definite separation and kept the tracing below the 10 cm. mark, obviating the use of a factor for calculating the area. The paper strips were dyed and fixed according to the method of Block, Durrum, and Zweig.[3] After exposure to ammonia fumes[2] the strips were scanned in the Spinco Analytrol. Chemical precipitation was accomplished by the Wolfson and Cohn[1] method utilizing the Reinhold[4] modification in which the precipitating solution is a sulfate-sulfite mixture instead of sodium sulfite. With each series of determinations, an aliquot of the control serum was run in duplicate with each method.

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†Becton, Dickinson and Company, Rutherford, N. J.

Commercially available human albumin (Cutter) and gamma globulin (Lederle) solutions were diluted with isotonic saline and various mixtures prepared; the total protein concentration was determined by micro-Kjeldahl analysis. Known quantities of the different protein solutions were added to a known serum to obtain the recovery data with the paper electrophoresis apparatus.

RESULTS

Fig. 1 presents the results of the comparison of the two methods of protein fractionation. In each portion of the figure the horizontal, solid lines signify the average value of the specific protein fraction expressed as per cent of the total protein concentration; the dashed lines represent the upper and lower confidence limits or ±2 standard deviations. Each dot is the average of duplicate determinations run on each serum.

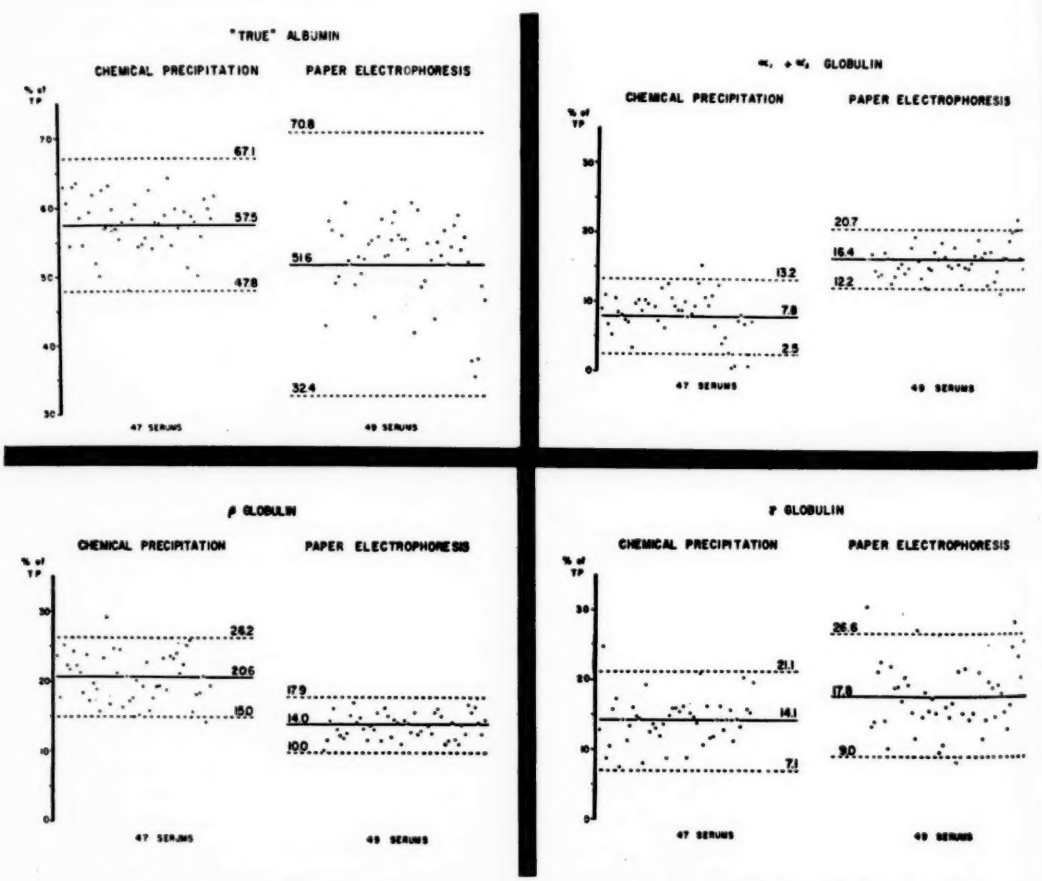

Fig. 1.—Comparison of serum protein fractionation by chemical precipitation (Wolfson and Cohn) versus paper electrophoresis (Spinco Model R). Solid horizontal lines represent the average value expressed as per cent of the total protein concentration. Dashed lines represent upper and lower confidence limits or ±2 standard deviations. Each dot represents the average of duplicate determinations on each serum.

The lower average serum albumin value determined by paper electrophoresis (Fig. 1) may be explained on the basis of the albumin tailing effect; the wider confidence limits may be due to a greater sensitivity of the method

and to structural changes in the protein which affect its affinity for the bromophenol blue dye. For example, the protein structure may have been altered during the passage of the blood through the tubing and needle of the collection set. The average total alpha globulin (Fig. 1) is twice as high with the paper electrophoresis method; some of this disagreement may be due to the fact that the alpha globulin is calculated by difference of two separate determinations in the chemical method. The average beta globulin (Fig. 1) is lower by paper electrophoresis. The value for beta globulin in the chemical method is obtained by adding the "true" albumin + alpha globulin and the gamma globulin fractions and subtracting the sum from the total protein; because of this, all the errors which may occur in obtaining the various fractions are summated in this beta globulin fraction.

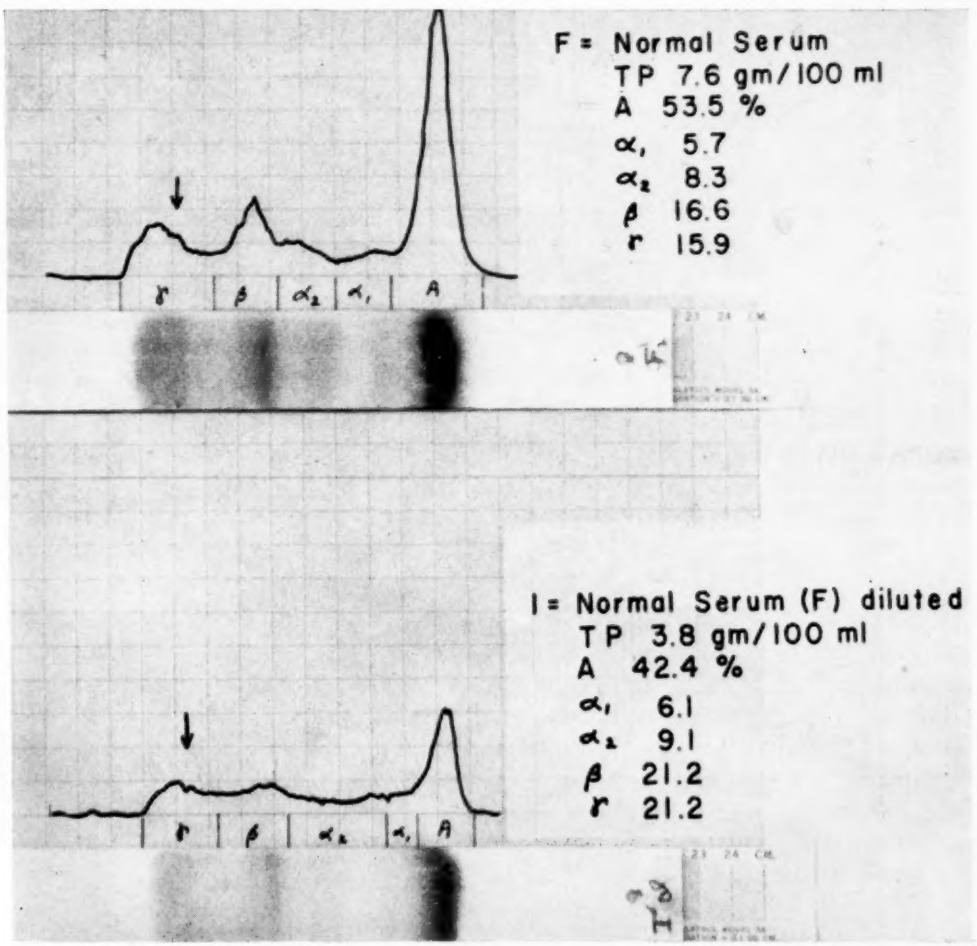

Fig. 2.—Typical electrophoretic patterns of normal serum obtained with Spinco Model R paper electrophoresis. Curve F is undiluted normal serum; Curve I is the same serum diluted 1:1 with isotonic saline. Line tracing is actual pattern obtained with Analytrol; the paper strip dyed with bromophenol blue dye is directly underneath. The arrow denotes the point of application of the 6 lambda of solution. TP equals total protein concentration in grams per 100 ml. A, α_1, α_2, β, and γ represent the specific albumin, alpha-1, alpha-2, beta, and gamma globulin fractions, respectively, in terms of per cent of total dye absorption.

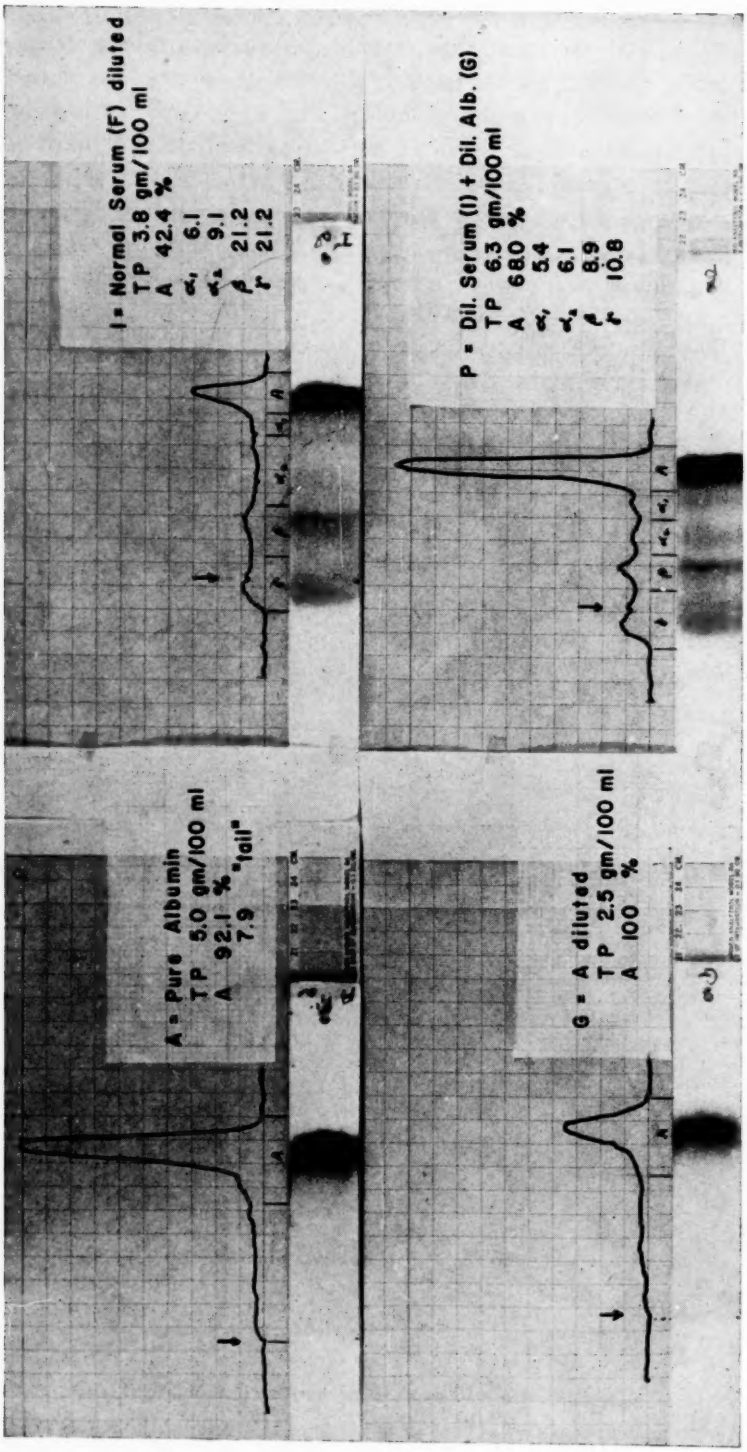

Fig. 3.—Typical electrophoretic patterns of albumin solutions. Curve A is pure albumin; Curve G is the same solution diluted 1:1 with isotonic saline; Curve I is diluted normal serum; and Curve P is the summation of Curves G and I. See Fig. 2 for explanation.

The average for the gamma globulin fraction (Fig. 1) is higher by paper electrophoresis. The difference may be due to some retention of dye by the albumin tail in the gamma globulin fraction. After the strips have been dyed and fixed, we have noted a dark line at the point of application of the sample; this may be denatured protein which does not migrate but does have a certain affinity for the dye, and thereby elevates the gamma globulin concentration in the final calculations.

Curve F in Fig. 2 illustrates the normal serum used in studying the recoveries of the various protein solutions. Dilutions of this normal serum with an equal volume of isotonic saline gave rise to Curve I. The low total protein concentration results in a low curve which is difficult to separate into its component fractions; the per cent composition is altered from the theoretical. Such alterations are prone to occur in solutions of low total protein concentrations since similar changes in the integration pattern of the Analytrol result in larger numerical changes in the per cent composition.

Fig. 3 illustrates a study with pure albumin. Curve A depicts the albumin solution at a concentration of 5.0 Gm. per 100 ml.; the tailing effect is calculated to be 7.9 per cent of the total dye absorption as computed with the Spinco Analytrol. Even though the line in Curve G is above the base line, nothing was picked up by the Analytrol so that the tailing effect is decreased in the diluted albumin solution with a total protein of 2.5 Gm. per 100 ml. Curve P illustrates a recovery experiment of adding albumin solution to normal serum. This was accomplished by diluting normal serum (Curve F, Fig. 2) with an equal volume of pure albumin (Curve A, Fig. 3); in essence this can be represented by Curve G + Curve I = Curve P. Fig. 4 illustrates a similar study with pure gamma globulin; no tailing effect is seen with the gamma globulin solutions (Curves B and H). Fig. 5 represents a typical study of one of the different mixtures of albumin and gamma globulin.

At a total albumin concentration of 5.0 Gm. or more per 100 ml., the tailing effect was found to average 5.7 per cent in 16 experiments. When the total albumin concentration was reduced in half, the average tailing for 16 experiments was only 2.8 per cent of the total area. This indicates that the concentration of protein in the specimen being analyzed will affect the amount of albumin tailing. Controlled conditions regarding albumin tailing and similar integration factors can and should be maintained by applying the same amount of protein (in our experiments approximately 60 mcg.) to the paper strip, no matter what the actual protein concentration in the specimen being studied. The average recovery, in 16 experiments, of pure albumin added to the normal serum was 90.8 per cent, and 105.2 per cent for gamma globulin.

DISCUSSION

Eriksen and associates[5] feel that varying amounts of the different protein fractions are carried over into other fractions during the chemical precipitation; alpha globulin is included with albumin, beta globulin is included with

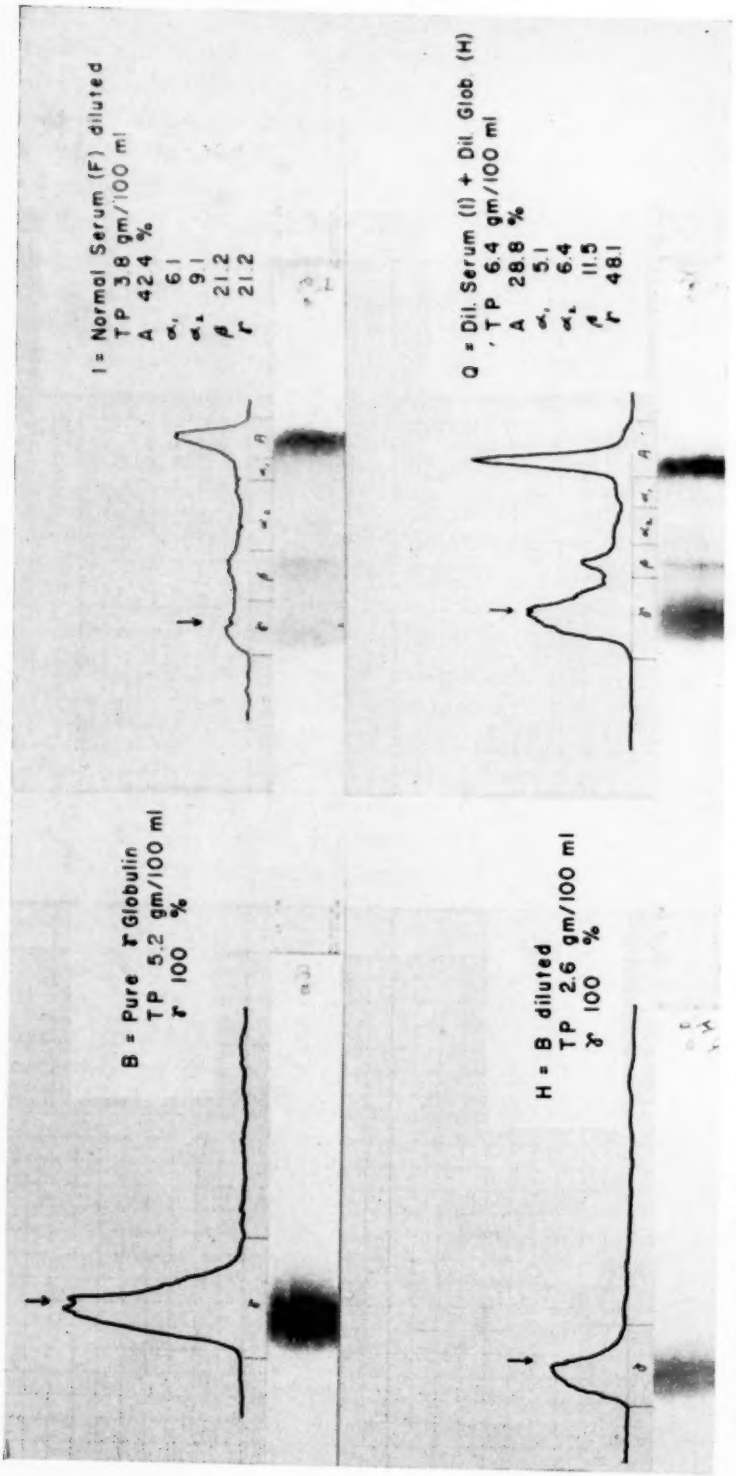

Fig. 4.—Typical electrophoretic patterns of gamma globulin solutions. Curve B is pure gamma globulin; Curve H is the same solution diluted 1:1 with isotonic saline; Curve I is diluted normal serum; and Curve Q is the summation of Curves H and I. See Fig. 2 for explanation.

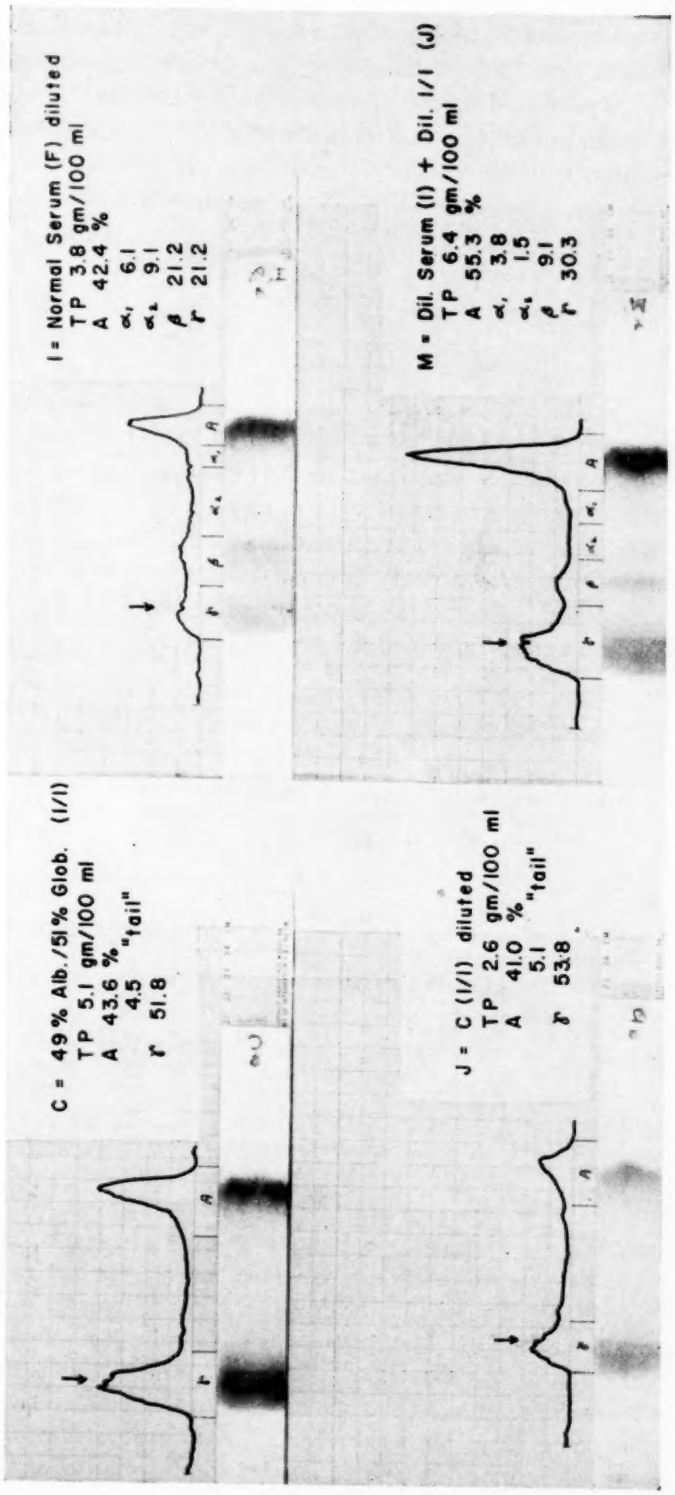

Fig. 5.—Typical electrophoretic patterns of 1:1 albumin/gamma globulin solution. Curve C is 49 per cent albumin and 51 per cent gamma globulin; Curve J is the same solution diluted 1:1 with isotonic saline; Curve I is diluted normal serum; and Curve M is the summation of Curves J and I. See Fig. 2 for explanation.

albumin + alpha globulin and with gamma globulin, etc. Thus, the determination of protein fractions by calculation of differences between appropriate quantities may be erroneous because of incomplete separation.

Our results indicate that the two methods of protein fractionation are not interchangeable although there is fairly close agreement between the values for albumin and for gamma globulin. This is not surprising since the two techniques are based on totally different principles—chemical precipitation by salt mixtures versus fixed electrophoresis on paper.

Table I lists representative data for the chemical fractionation of serum proteins by the Wolfson and Cohn[1] method or its modifications and paper and/or free (moving boundary) electrophoresis. The original data of Wolfson and Cohn[1] are based on 4 pools of serum collected from 50 to 150 blood donors. Ellerbrook's[6] larger series of data on normal individuals is in close agreement with our data on professional blood donors. The serum protein fractions by paper electrophoresis show a range of values which may be influenced by the source of donors and the number of specimens studied (Table I). It is obvious that even among the free electrophoresis data there is a wide range of values. The data of Reiner and co-workers[7] illustrate differences in the results obtained on professional versus family blood donors (Table I).

TABLE I. COMPARISON OF REPRESENTATIVE DATA OF PROTEIN FRACTIONATION

INVESTIGATORS	SOURCE* OF SERUM	METHOD OF† FRACTIONATION	NO. OF SPECIMENS	PER CENT COMPOSITION					
				ALBUMIN	GLOBULIN				
					α_1	α_2	TOTAL α	β	γ
Present authors	PD	C	47	57.5			7.8	20.6	14.1
Ellerbrook[6]	N	C	74	58.5			11.1	15.9	14.5
Wolfson et al.[1]	NP	C	4	53.8			15.7	13.4	17.1
Present authors	PD	PE	49	51.6	5.8	10.6	16.4	14.0	17.8
Klatskin et al.[10]	N	PE	10	58.6	3.4	8.0	11.4	12.5	17.6
Knights et al.[11]	N	PE	20	50.0	5.3	9.6	14.9	14.5	20.6
Verghese et al.[12]	N	PE	20	54.0	5.3	8.3	13.6	12.0	20.3
Klatskin et al.[10]	N	FE	10	57.7	4.5	7.2	11.7	15.3	15.3
Gordon[13]	N	FE	37	56.3	5.7	9.3	15.0	14.7	14.1
Reiner et al.[7]	PD	FE	20	53.6	7.5	9.0	16.5	14.1	15.7
Reiner et al.[7]	FD	FE	60	56.8	7.2	8.7	15.9	12.8	14.4
Ricketts et al.[14]	N	FE	10	60.3	4.0	9.7	13.7	12.8	13.2

*PD = Professional Donors. N = Normal Individuals. NP = Normal Pools (50 to 150 donors per pool). FD = Family Donors.

†C = Chemical Precipitation. PE = Paper Electrophoresis. FE = Free Electrophoresis.

Paper electrophoresis and moving boundary electrophoresis do not give exactly similar analytic data[8]; in the latter case the results are expressed in terms of refractive index, whereas in the former they are usually expressed in terms of dye binding capacity, which in the case of acid dyes is largely an expression of the number of basic groups. This dye affinity is subject to many variables, such as method of coagulation of the separated proteins, protein structural changes, type of paper used, etc. The differentiation of the fractions on the tracing made with the Spinco Analytrol, especially for the alpha-1

and alpha-2 fractions, is subjective. An electrophoretic peak does not necessarily represent a single, homogeneous protein, but may consist of a group of heterogeneous proteins having a similar electrophoretic mobility. The fractions obtained by paper electrophoresis are per cent of the total dye uptake, which we assume to represent the total protein content; we feel that it is erroneous to convert such fractions into terms of Gm. per 100 ml. All these factors lead us to agree in principle with the work done by Polimeni and Turitto[9] who designate the separated fractions by Roman numerals rather than protein terms. We feel, however, that for clinical purposes the components should still be called albumin, alpha-1 globulin, etc.

Because of the conditions of our study—the use of professional donors, the method of collection, and the overnight refrigeration of the serum prior to analysis—we feel it is not warranted to list the values obtained by the two methods as normals for serum protein fractionation. Rather, we have presented a *comparison* of the results between chemical and paper electrophoretic separation of the serum protein fractions.

SUMMARY AND CONCLUSIONS

Our comparison of protein fractionation by the salt precipitation method and by paper electrophoresis showed that the results obtained by the two methods are not interchangeable. This emphasizes the fact that each laboratory must always establish normal values for the conditions under which each method is used.

The total protein concentration in the specimen to be analyzed by paper electrophoresis is very important because (a) it affects the degree of albumin tailing, (b) the integration patterns with the Spinco Analytrol at low protein levels are not as reliable as those with normal total protein concentration, and (c) low total protein concentration results in poor separation of the globulin fractions.

Such conditions should be controlled by adding a relatively similar amount of protein to the paper strip no matter what the actual concentration of total protein in the serum specimen. A known serum should be run with each set of unknowns.

We wish to thank Dr. Kurt Stern and his staff of the Blood Bank at the Mount Sinai Medical Research Foundation and Hospital for their cooperation in obtaining the blood specimens.

Thanks are due Mr. Richard Bass for technical assistance.

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